Identification and characterization of novel positive regulators of the Target of Rapamycin (TOR) pathway in *Drosophila melanogaster*

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

Pankuri Goraksha-Hicks

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Dr. Thomas P Neufeld

December 2008

ACKNOWLEDGEMENTS

It is my pleasure to thank the individuals who have made this thesis possible.

I am deeply grateful to my adviser, Dr. Tom Neufeld, for being an excellent mentor throughout my graduate training. He provided me with encouragement, sound advice and the confidence to believe in myself and my work when things were not going as planned. He shared with me his expertise, training, and was always available for discussions, for which I am greatly indebted to him.

I would like to thank Dr. Kun-Liang Guan and Dr. E. Kim for sharing and exchanging scientific ideas, which resulted in a fruitful publication and contributed to this thesis.

I am thankful for the support of my very understanding committee members and for their valuable time and insight into my research progress – Dr. Mike O'Connor, Dr. Do-Hyung Kim, Dr. Alex Lange and Dr. Jeff Simon.

I wish to express gratitude to my parents-in-law, Tom and Carol Hicks, for not only opening the comforts of their home, but also for providing excellent company, constant support, and emotional sanity through all the late nights and hurried days as I wrote this thesis.

I cannot imagine the culmination of my graduate career and this thesis without the support and encouragement from my beloved husband, Caleb Hicks, who spent many hours earnestly listening to all my research ideas, successful updates, and tales of impatience even when they did not make sense to him. I deeply appreciate his patience and caring as I went through the ups and downs of science and exams.

Most importantly, I am forever grateful to my parents - Uttam and Swati Goraksha. They raised me, loved me, educated me and gave me the independence and confidence to pursue my scientific dreams. To them I dedicate this thesis.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER 1: INTRODUCTION

I. Importance of TOR-mediated growth

The biological process of growth, which results in mass accumulation thereby increasing the size of cells, organs, and eventually the organism, is critical for the normal development and functioning of a living organism. In order to achieve optimum growth and survive favorably, organisms must be able to sense and respond to both genetic and environmental cues and adapt their physiological and developmental decisions accordingly. In eukaryotes, the Target of Rapamycin (TOR) signaling pathway plays a critical and central role in coupling the processes of cell growth and proliferation by virtue of its ability to balance growth with nutrient and energy availability. TOR regulates growth by upregulating processes that lead to mass accumulation such as cell division, transcription, translation and protein synthesis, and downregulating catabolic processes such as autophagy. TOR, a serine-threonine protein kinase of the phosphatidylinositol-3-kinase-related kinase (PI3KK) family, functions as the main player of this ancient signaling pathway that is conserved across all eukaryotic species such as yeast, *Dictyostelium*, *C. elegans*, *Drosophila*, *Arabidopsis* and mammals.

TOR kinase was first identified in yeast and named as Target of Rapamycin for a natural antibiotic derived from *Streptomyces hygroscopicus* that can restrict growth via cell-cycle arrest (Heitman J et al., 1991). This growth arrest was achieved by binding of rapamycin to an endogenous protein FKBP12.

The direct binding of the resulting rapamycin-FKBP12 complex to TOR kinase could possibly disrupt the structural integrity of TOR protein itself or interfere with its subsequent interactions with other proteins thereby interfering in normal growth. Yeast contains two TOR genes while metazoans have one TOR gene. Regardless, TOR exists in two different protein complexes TOR complex 1 and 2 (TORC1 & TORC2) that are differentially regulated and differ in their sensitivity to rapamycin. Exercising distinct functions through these two complexes, TOR acts as a master switch for temporal and spatial growth via regulation of growth homeostasis between anabolic and catabolic processes on nutrient stimulation (Figure 1A).

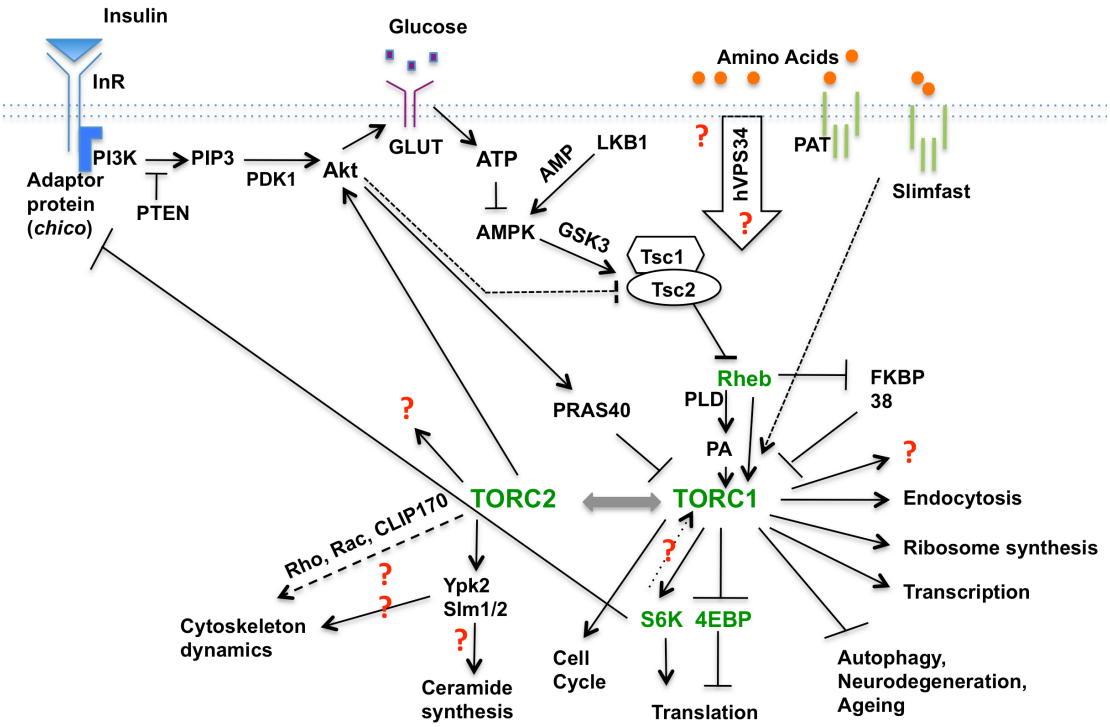


Fig. 1A. A summary of the TOR network

TOR kinase functions as the core player in this master regulatory pathway that connects nutrient, growth factor and energy availability to enhance organismal growth and size by functioning in two different complexes, TORC1 and TORC2. TOR integrates these upstream signals via the negative regulator of the pathway, TSC complex, which inhibits TORC1 by inactivating Rheb. While TORC1 mediates the temporal aspects of growth to regulate cell cycle, translation & protein synthesis, autophagy, ageing and neurodegeneration, transcription, ribosome biogenesis and endocytosis, TORC2 regulates the spatial aspects of growth via controlling cytoskeleton organization, ceramide synthesis and through phosphorylation of Akt kinase. There are a number of unanswered questions and connections in the pathway, highlighted by the question marks while dashed lines denote unclear relationships and mechanisms. Some of the regulators and effectors of TORC1 and TORC2 are still unknown. The regulation of cytoskeleton organization by TORC2 is not well understood. There exists a regulatory feedback between the TOR pathway and Insulin signaling, as depicted, that adds to the complexity. The most long-standing question in the field is how amino acids are sensed in the pathway and which regulators are involved. hVps34, though identified as a possible candidate in humans for amino acid sensing, has not been validated for this important function in other systems.

II. TOR acts as an evolutionary conserved regulator of growth and development on nutrient stimulation.

TOR regulates a variety of cellular growth processes by acting as a nutrient, energy and growth factor sensor to modulate cell growth and proliferation. *Drosophila* TOR mutants exhibit a cell autonomous decrease in cell size, endoreplicative cell cycle arrest at the G0/G1 boundary and reduced phosphorylation of its downstream effectors of translation, S6K and 4EBP (Oldham S et al., 2000, Zhang H et al., 2000). These mutants mimic amino acid starvation by exhibiting growth arrest at second instar larval stage, decrease of nucleolar size and aggregation of lipid vesicles in the fat body. Yeast mutants for TOR also phenocopy starvation (Rohde JR et al., 2008).

Recent studies show that TOR activity is not only important for cell growth, proliferation and differentiation but also in a developmental context. TOR mutants in *Drosophila* and *Arabidopsis* arrest at early developmental stages (Oldham S et al., 2000, Zhang H et al., 2000, Menand B et al., 2002). Knockdown of TOR in *C. elegans* genetically or by using RNAi results in intestinal degeneration and growth arrest (Long X et al., 2002). Zebrafish TORC1 was also found to be important in the developmental context for epithelial morphogenesis in vertebrate intestine (Makky K et al., 2007). One shared process during development and metastasis is epithelial to mesenchymal transition (EMT), which enhances cell

motility and invasiveness. It has recently come to light that TGF-ß induces this process by increasing translation through activation of Akt-mTOR in a rapamycin-sensitive manner (Lamouille S & Derynck R, 2007). This example of mTOR playing an important functional role downstream to TGF-ß in a developmental context can have important implications that need to be investigated. The developmentally important regulator, Wnt, also crosstalks with mTOR. GSK3, a Wnt-downstream effector, phosphorylates and activates Tsc2, a negative regulator of TOR, thereby inhibiting mTOR activity (Inoki K et al., 2006). This relationship has potential for developmental regulation via Wnt-mediated TOR signaling.

The role of InR (Insulin Receptor)/TOR signaling in regulation of developmental timing has also been studied. In *Drosophila*, the posterior to anterior progression of the morphogenetic furrow results in a similar wave of neuronal differentiation in the eye imaginal disc in third instar larvae. It is observed that activating InR/TOR pathway results in precocious neuronal differentiation without changes in the cell fate (Bateman JM and McNeill H, 2004). This effect of InR/TOR mediated differentiation timing was found to be tissue specific.

TOR may also play a role in sexual differentiation. Hypomorphic *Drosophila* TOR female mutants undergo defective oogensis and are sterile

though viable, similar to S6K mutants (Zhang Y et al., 2006, Radimerskii T et al., 2002).

Thus it is evident that intricate developmental decisions may also be coordinated by TOR pathway along with nutrient-mediated cell and tissue growth. This aspect of the TOR pathway is completely unclear. Uncovering novel regulators and effectors of TOR signaling can further our understanding of TOR network in a developmental context.

III. TOR complex formation

The key player in this pathway is the evolutionary conserved PI3KK family member protein TOR. TOR is a relatively large 280 kD serine/threonine protein kinase that contains the conserved PI3 kinase homology domain adjacent to the rapamycin-binding FRB domain and a FATC domain that is implicated in sensing the redox status of the cell at its C-terminal (Dames SA et al., 2005). The central region contains a FAT domain of unknown function. The amino terminal of the TOR protein is characterized by the presence of a number of tandem HEAT repeats. TOR functions as a multimeric protein and mutations in the HEAT, FRB, FATC and kinase domain disrupt TOR activity (Zhang Y et al., 2006).

The versatility of TOR kinase is achieved by its ability to bind to other proteins and form complexes, which functionally coordinate and integrate multiple cellular signaling pathways. TOR exists in two complexes called TORC1 and TORC2 [Fig. 1B]. In metazoans, these two complexes differ in their sensitivity to

rapamycin and their components other than TOR, thereby maintaining specificity of signaling outputs. TORC1 is made up of TOR, Raptor (Regulatory Associated Protein of TOR), GßL/LST8 and PRAS40 (Proline rich Akt substrate 40). Raptor binds to the superhelical structural motifs arising in the HEAT repeats in mTOR and acts as a scaffold to recruit downstream targets S6K and 4EBP (Kim DH et al., 2002, Nojima H et al., 2003). Endogenous PRAS40 binding to mTORC1 is inhibitory in nature and is relieved by insulin stimulation (Wang L et al., 2007). PRAS-40 binds to mTORC1 via Raptor and is thought to hinder binding of mTORC1 to its substrate 4EBP (eIF4E-binding protein) (Wang L et al., 2007). PRAS40 is also phosphorylated by mTOR at three sites as determined by two dimensional phosphopeptide mapping and mutational analysis (Wang L et al., 2008). By binding to the kinase domain, GßL enhances its catalytic activity (Kim DH et al., 2003). The catalytically active kinase domain of TOR also binds to Rheb, the small GTPase that is the best elucidated upstream positive regulator of TOR and is subject to inhibition via TSC2-GAP activity (Long X et al., 2005, Zhang Y et al., 2003).

Studies demonstrate that TORC1 is rapamycin sensitive and is specifically involved in nutrient-mediated regulation of autophagy, translation and protein synthesis. The two well-known downstream targets of TORC1 in metazoans are S6K and 4EBP, both of which are important for cell mass accumulation via affecting translation and protein synthesis. TORC2, consisting of TOR, Sin1,

Rictor (Rapamycin insensitive companion of TOR) and GßL, is a recent arrival on the stage as the elusive PDK2 important for phosphorylation of Akt at Ser 473 (Sarbassov DD et al., 2004, Sarbassov DD et al., 2005). Sin1, Rictor and GßL each are required for mTORC2 activity (reviewed in Bhaskar PT and Hay N 2007). TORC2 also phosphorylates PKC and Akt at the TM (turn motif) and HM (hydrophobic motif) (Ikenoue T et al., 2007) thus promoting their activity. A conserved role for TORC2 in yeast and mammals is for the regulation of actin and cytoskeleton dynamics (Sarbassov DD et al., 2004, Jacinto E et al., 2004). Mutations in either Rictor (Sarbassov DD et al., 2005) or Sin1 (Jacinto E et al., 2004) result in mTORC1 activation suggesting competition between the two complex members for mTOR binding. TORC2 though resistant to acute rapamycin treatment becomes sensitive after prolonged exposure to rapamycin, resulting in a progressive loss of Rictor-mTOR association depending on the cell type (Sarbassov DD et al., 2006).

To summarize, it has now been elucidated that while TORC1 is involved in the temporal aspects of growth, proliferation and development via affecting translation and protein synthesis, TORC2 regulates the spatial aspects through regulation of actin cytoskeleton organization.

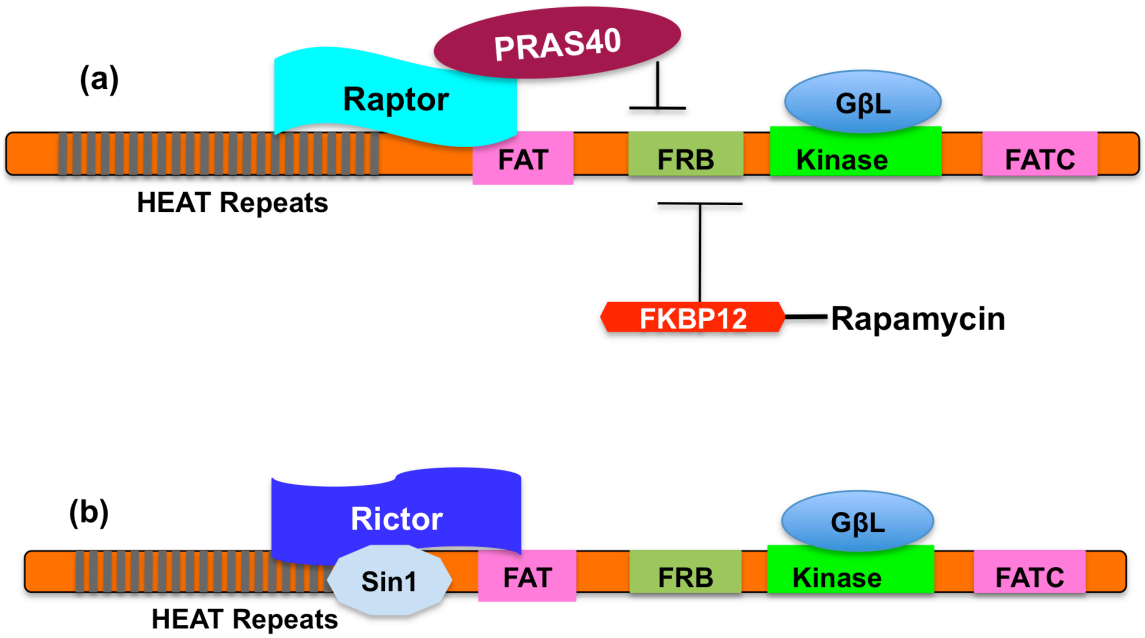


Fig1B. Schematic of the two TOR complexes and their compositions

TOR carries out pleiotropic downstream effects by existing in two distinct complexes, TORC1 and TORC2. The domain structure of the TOR protein itself from amino to carboxy terminal direction is as follows: HEAT repeats, important for protein-protein interaction; FAT, a domain conserved in al PI3KK family members; FRB, the docking site for FKBP12-rapamycin complex; Kinase domain that is required for catalytic action and FATC, a domain that is important for sensing redox state and needed along with FAT domain for kinase activity. (a) Protein components of the TORC1 complex, which is rapamycin-sensitive and promotes temporal growth. (b) Protein components of the TORC2 complex, which is rapamycin-insensitive and promotes spatial growth.

The exact mechanisms and stimuli that influence the TOR-complexes that get activated and regulated remain to be understood and now contribute towards the complexity of TOR-mediated regulation and versatility. It has been observed that the two well known upstream regulators of TOR, TSC complex and Rheb, exercise opposing effects on TORC1 and TORC2 (Yang Q et al., 2006). The identification of the two TOR complexes with unique binding partners illustrates that TOR pathway achieves multiple signal coordination and relay through a number of proteins, some of which are still unidentified. As of now, a readout for TOR kinase activity comes in the form of phosphorylation of two well-known downstream targets, S6K and 4EBP, by TORC1 and phosphorylation of the pro-survival kinase Akt by TORC2 in higher eukaryotes. In order to dissect the complexity of the TOR pathway, there is a need to identify and characterize novel regulators and targets of TOR.

The nutrient-mediated stimulation of TOR activity in metazoans occurs at three levels – availability of growth factors like insulin, availability of glucose, which translates into the energy status of the cell and amino acid availability. The stimulation of TORC by insulin signaling and glucose has been well elucidated and will be discussed later in this chapter. However, there is no clear knowledge of the regulators involved in amino acid sensing. The precise sensor of amino acid in the TOR pathway has remained elusive for a long time. Recently hVps34 has been indentified as a candidate to satisfy amino acid sensing for mTOR

signaling (Nobukini T et al., 2005). However, this still remains a much-debated question in the field of TOR signaling.

IV. Upstream regulators of TOR activity

TOR pathway assimilates signals from cellular energy and stress levels and ties them to growth factor and amino acid availability to bring about coordination of cellular metabolism, resulting in temporal and spatial cellular growth. Within the core TOR pathway, studies of the negative regulators Tsc1/2 and the positive regulator Rheb, a small GTPase, have been the most illuminating. Discussed below are some of the known regulators of TOR activity.

a. TSC complex

The Tuberous Sclerosis complex was identified in 1993 to consist of two tumor suppressor genes, *Tsc1* and *Tsc2*. A mutation in either Tsc1, known as hamartin, or Tsc2, known as tuberin, results in a dominant genetic disorder called Tuberous Sclerosis, that has been described earlier.

Different groups independently identified the TSC complex (Tuberous Sclerosis) as the negative regulator of PI3K-Akt mediated growth (Gao X and Pan D, 2001, Potter CJ et al., 2001, Tapon N et al., 2001). A mutation in the *Tsc1* gene resulted in overgrowth of organ and cell size in *Drosophila*. In accordance, co-overexpression of Tsc1 and Tsc2 resulted in reduced cell growth and proliferation. The ability of Tsc1 and Tsc2 to occur in a complex to achieve growth inhibitory effects was also demonstrated. Tsc2 functions as a GAP

(GTPase Activating protein) while Tsc1 is attributed the function of maintaining complex stability (Zhang Y et al., 2003).

Definitive studies about their exact relationship in the pathway and mechanism of action were shown subsequently (Inoki K et al., 2002, Gao X et al., 2002, Potter CJ et al., 2002). Genetic analyses in *Drosophila* showed that Tsc1-2 act to inhibit S6K and 4EBP-phosophorylation, antagonizing TORC1 activity. Biochemical experiments provided evidence that in both *Drosophila* and mammalian systems *in vitro* and *in vivo*, phosphorylation by Akt at two target residues within Tsc2 inhibits phosphorylation of TOR-targets S6K and 4EBP. Loss of Tsc1-Tsc2 increased S6K phosphorylation, rendering cells insensitive to amino acid withdrawal. This led to the speculation that Tsc1-2 maybe the elusive sensors for amino acid. This issue was put to rest when studies demonstrated that in the absence of Tsc2 amino acid withdrawal still causes dephosphorylation of TOR targets (Smith EM et al., 2005). Nonetheless, in lieu of the absence of Tsc1-2 homologs in yeast, it seems unlikely that they act as amino acid sensors since amino acid sensing is the most conserved function of the TOR pathway maintained during evolution. On the other hand, this affords TSC the unique position as integrator, gatekeeper and transducer of various signals like growth factors, energy levels, and cellular stress to the downstream TOR pathway that arose as multicellularity evolved.

In agreement with this, as discussed later in this chapter, we find that indeed most of these signals are transduced at the TSC level. In addition to the above signals, Tsc2 also integrates signals from MAPKs such as Erk1 and Rsk1. Using mass spectrometry and immunoblotting analysis of phosphorylation events, TSC1 and TSC2 were identified as critical for ERK1 and RSK1 (p-90 ribosomal S6 kinase) activation in mammalian cells (Ballif BA et al., 2005). Activated ERK1 phosphorylates TSC2 at Ser 1798 residue leading to mTORC1 inactivation, similar to that seen in presence of tumor promoting phorbol esters (Roux PP et al., 2004). A separate study also found that ERK-dependent Tsc2 phosphorylation lead to disassembly of the TSC complex resulting in mTORC1 activation (Ma L et al., 2005).

One way that TSC can integrate these signals for TORC1 regulation is by association with 14-3-3, a highly acidic dimeric protein that regulates proteins by modulating their subcellular localization. Generally, under low nutrient conditions Tsc2 is hypophosphorylated and exists in a complex with Tsc1 at the cell membrane inhibiting mTORC1 via exhibiting GAP activity towards Rheb. On growth factor stimulation in mammalian cells, Tsc2 gets phosphorylated by Akt, goes to the cytosol where it binds to 14-3-3 on its two Serine residues resulting in seclusion from farnesylated Rheb (Cai SL et al., 2006). This is a good example of how TOR activity can be altered by membrane partitioning and subcellular localization, which will be discussed towards the end of this chapter.

b. Rheb

Rheb (Ras homologue enriched in brain), a member of the Ras subfamily of small GTPases, is conserved for nutrient-mediated growth regulation from yeast to mammals. Soon after the knowledge about the involvement of TSC in modulating mTORC1 targets, it was reasoned that the TOR activation might involve a GTPase. Genetic screening in *Drosophila* by three independent groups identified Rheb as the potential small GTPase that promotes growth and cell proliferation via TOR activation (Saucedo LJ et al., 2003 NCB, Stocker H et al., 2003, Patel PH et al., 2003). Both genetic and biochemical analyses demonstrate that Rheb functions downstream to Tsc2 and upstream of TOR to positively affect nutrient-mediated growth. Overexpression of Rheb results in overgrowth due to increase of cell and organ size but not of cell number, achieved by driving cells from G1-S phase. Mutations in TOR inhibit growth stimulatory effects of overexpressed Rheb. Rheb was shown to be an active GTPase and its nucleotide status was shown to be important for its subsequent S6K activation and growth promoting effects.

Since Rheb growth stimulatory effects could not be affected by starvation in the above studies (Saucedo LJ et al., 2003, Stocker H et al., 2003), it was speculated that it might be the elusive amino acid sensor in the TOR pathway. Contrary to this hypothesis, RNAi studies in *Drosophila* S2 cells have now definitively proven that Rheb as well as TOR do not increase direct uptake of bulk

amino acids, glucose or arginine (Hall DJ et al., 2007). Rheb overexpression has little effect on the intracellular steady state of individual amino acids in mammalian cell culture. Hyperactivation of Rheb does not change the decrease seen in intracellular amino acid pool upon extracellular amino acid withdrawal (Nobukini T et al., 2005).

Concurring to its GTPase nature, Rheb gets farnesylated and exists in either an inactive GDP or an active GTP bound state. Rheb farnesylation when impaired leads to a cell cycle arrest at the G0-G1 boundary in yeast, similar to that seen in TOR mutants (Yang W et al., 2001), suggesting its importance for TOR activation. Rheb can stimulate TORC1 activity indirectly via inhibiting FKBP38, a newly indentified mTORC1 inhibitor in a GTP-dependent manner (Bai X et al., 2007). Rheb directly bound to FKBP-38 via its switch 1 region (Ma D et al., 2008). Under nutrient limitation FKBP38 binds to mTOR in the absence of Rapamycin. On nutrient and growth factor stimulation, activated Rheb can antagonize FKBP38 mediated mTORC1 inhibition.

It has also been shown that Rheb directly binds to mTORC1 via a small motif in the kinase domain of mTOR and also via binding to LST8 (Long X et al., 2005). Though direct binding of Rheb to mTOR occurs in a nucleotide-independent manner, it is sensitive to GTP levels. Surprisingly, though nucleotide-deficient Rheb mutants can bind to mTOR, the kinase level of mTOR within such a complex is assayed to levels similar to kinase dead mTOR. This

implies that the GTP-bound state of Rheb is required for catalytic capability of mTORC1.

In *Drosophila*, genetic and biochemical analyses recently identified TCTP (Translationally Controlled Tumor Protein) as a GEF (Guanine exchange factor) for the small GTPase Rheb (Hsu YC et al., 2007). *TCTP* mutants phenocopy *Rheb* mutants, showing reduced cell growth, cell number and cell size. Both human and *Drosophila* TCTP can mediate GDP/GTP exchange on the corresponding Rheb protein. However, TCTP could not be validated as a bona fide positive regulator of mTORC1 in subsequent mammalian cell culture experiments by other investigators (Rehmann H et al., 2008, Wang X et al., 2008). TCTP did not interact with Rheb or mTORC1 by immunoprecipitation or NMR spectroscopic studies, no GTP-exchange between TCTP and Rheb was observed and TCTP depletion did not reduce mTORC1 signaling as measured by S6K and 4EBP-phosphorylation. Thus it is doubtful that TCTP is a Rheb-GEF leaving the stage open for a new candidate.

Questions about the exact mechanism of Rheb-mTOR interaction are still unclear even though subsequent mutagenesis studies have shown that the Switch 2 region of Rheb, which is not altered by GTP loading, and the Switch 1 region, which is highly altered by GTP loading, are needed for direct interaction with mTOR (Long X et al., 2007, Long X et al., 2005). The observation that amino acid withdrawal affects Rheb-mTOR interaction (Long X et al., 2005) needs to be

validated and the mechanism behind it needs to be elucidated. This will shed light on the amino acid sensing mechanism within the TOR pathway, which based on all the above studies functions upstream of Rheb-mTORC1 interaction. In addition, the candidate for a Rheb-GEF especially in the mammalian system is still missing.

c. Amino acids

The amino acid sensing attribute of the TOR pathway is perhaps its most ancient aspect, being conserved from unicellular to multicellular eukaryotes. The first link pointing to a direct input of amino acids for activating TOR pathway came from mammalian cell culture studies. It was shown that removal of amino acids from cell culture media for short time intervals of less than 2 hours dephosphorylated TOR translational targets (S6K and 4EBP) and rendered them unresponsive to insulin action (Hara K et al., 1998). Since S6K-phosphorylation is sensitive to amino acids, we can deduce that TORC1 is more sensitive to amino acid modulation and sensing as compared with TORC2. Indeed, TSC and Rheb both affect TORC1 and TORC2 differently as mentioned earlier.

A rapamycin-resistant S6K1 mutant is also resistant to amino acid withdrawal, suggesting that amino acid sensing lies at the level of mTORC1 or upstream to it (Hara K et al., 1998). A number of studies have now shown that amino acid sensing in the TORC1 pathway is largely independent of PI3K and Rheb-GTP loading (reviewed in Avruch J et al., 2008). In spite of many studies

concluding the importance of amino acids as modulators of TORC1, whatever little is known about which players are chiefly involved and mechanisms for the same are quite debatable.

Based on the sensitivity of the structural interactions within TORC1 formation, it has been suggested that raptor-TOR itself is the nutrient sensing component (Kim DH et al., 2002). However, based on other *in vitro* kinase assays carried out by withdrawal and re-addition of amino acids, TORC1 stability does not seem to be regulated by amino acid (reviewed in Avruch J et al., 2008).

In yeast, a genome wide screen for identification of genes that exhibited synthetic lethality or reduced fitness with *tor1* led to the identification of a number of components of the Class C Vps Complex (Zurita-Martinez et al., 2007), including *Vps34* and *Vps15*. Addition of rich nitrogen sources such as glutamate or glutamine rescues the growth defects seen in individual *vps* mutants as well as in a t*or1pep3* temperature sensitive mutant. This suggests that an intact Class C Vps complex is required for amino acid homeostasis and subsequent efficient TORC1 signaling for growth in yeast. Recently, *hVps34* (encoding Type III PI3K) was also identified as a candidate for amino acid sensing based on the decrease and increase of S6K-phosphorylation seen on depletion and overexpression of hVps34 respectively, along with its kinase-like partner hVps15 (Nobukini T et al., 2005). Amino acids lead to increased $Ca2^{+}$ levels through unknown mechanisms, which bind to and stimulate hVps34 kinase activity leading to recruitment of PI3P

(Gulati P et al., 2008). In contrast, genetic mutations in *Drosophila Vps34* do not show any changes in TORC1 activity. In *Drosophila*, *Vps34* promotes endocytosis and autophagy. Under starvation conditions, TOR-Atg1 signal recruits Vps34 activity to the autophagosomal membrane (Juhasz G et al., 2008). Similarly, in *C.elegans* RNAi knockdown of CeVps34 and CeVps15 does not recapitulate CeTOR or CeRaptor deficiency (Aruch J et al., 2008). It is possible that the effects seen with *hVps34* occur only in the mammalian system because of production of PI-3-P.

A suppression of t-RNA aminoacylation, by using inhibitory chemicals like amino acid alcohols, cyclohexamide or puromycin and genetic mutations, also inhibits S6K activity (Iboshi Y et al., 1999). This implies that the yet unidentified sensor could detect amino acid presence indirectly by perceiving the levels of uncharged t-RNAs. The missing identity of such important regulators of TOR, once again underscores the need to implement strategies for dissecting the TOR network using available genetic screening and protein-protein interaction approaches.

d. Insulin signaling

TOR is conserved among all eukaryotes implying the importance of this ancient nutrient-sensing pathway. TOR is not only important for cell-intrinsic growth but is important in communicating and relaying nutritional response. In order to maintain this homeostasis, TOR crosstalks with growth factor induced

pathways that have evolved as a result of eukaryotic multicellularity, particularly Insulin and Insulin-like peptides signaling.

Secreted insulin/insulin-like peptides act as a ligand and bind to the insulin receptor at the cell surface leading to recruitment, phosphorylation and activation of Insulin Receptor Substrate 1 (IRS1), which in flies is encoded by *chico*. IRS1 along with p60 subunit recruits phosphatidylinositol-3-kinase (PI3K) to the cell membrane. Once at the cell membrane, PI3K phosphorylates phosphotidylinositol-4, 5-bisphosphate (PIP_2) at the third position producing the important messenger phosphotidylinositol-3, 4,5- triphosphate (PIP_3).

High levels of PIP_3 result in the membrane recruitment of pleckstrin homology (PH) domain-containing proteins like phosphoinositol-dependent kinase 1 (PDK1) and the prosurvival protein Akt kinase. At the cell membrane, Akt is activated on phosphorylation by PDK1 and TORC2. Akt-activation as a result of the canonical PI3K signaling is important for the well-known PI3K functions of modulating survival, growth, metabolism and glucose homeostasis (reviewed in Carracedo A and Pandolfi PP, 2008). The tumor suppressor PTEN (phosphatase with tensin homology), a gene mutated with high frequency in cancers, is an important regulator of reversal of PIP_3 to PIP_2 and its malfunction promotes tumorogenesis.

Activated Akt activates TORC1 in two ways. First, it phosphorylates Tsc2 thereby inactivating it and inhibiting its GAP activity towards the small GTPase

Rheb (Inoki K et al., 2002, Potter CJ et al., 2002). Secondly, Akt phosphorylates PRAS40 (proline rich Akt substrate of 40kDa), which normally binds to Raptor inhibiting mTORC1activity (Sancak Y et al., 2007). By relieving the PRAS40-Raptor-mTOR interaction, mTORC1 is activated. Akt also promotes translocation of glucose transporters to the cell membrane, which eventually promotes TOR activity through increased energy status of the cell. Two key outcomes of the canonical PI3K-signaling as exerted by the kinase activity of PDK1 and Akt are activation of S6K, a downstream TORC1 target, and the inhibition of the transcription factor FOXO, respectively (Frodin M et al., 2002, Lizcano JM et al., 2003). This allows a third mechanism for Akt-activation of TORC1 activity since it has been demonstrated that FOXO negatively inhibits transcriptional activation of Raptor, a TORC1 member (Jia K et al., 2004).

The significance of Tsc2 phosphorylation by Akt is different in *Drosophila* versus mammalian system. Inoki et al., 2002, demonstrated that exposure to the kinase activity of Akt produced a slower migrating Tsc2 suggesting modification by phosphorylation. However, subsequent studies using non-phosphorylatable or phospho-mimicking mutations demonstrate a complete rescue of Tsc2 null animal lethality (Dong J and Pan D, 2004), proposing that Tsc2 may not be a critical target of Akt-phosphorylation in *Drosophila*. With the pleiotropic downstream effects for regulation of diverse biological processes exercised by both PI3K and TOR, it is not surprising that there may exist feedback and direct

cross talk between these key pathways, which will be addressed at the end of this chapter.

e. Energy

The close connection between the nutrient status of the cell and translational capacity is not new. In yeast where glucose acts as a signaling molecule, its depletion inhibits protein synthesis independently of TOR inactivation (Ashe MP et al., 2000). This inhibition is post-transcriptional and in part due to the altered energy status of the cell. The resulting imbalance between ATP and AMP ratios due to glucose deprivation turns on the kinase AMPK in all eukaryotes from yeast to mammals. In higher eukaryotes, the cellular energy status sensor AMPK can also be activated by stress that decreases ATP levels such as hypoxia and muscle contraction. AMPK activity is induced by phosphorylation of LKB1, a tumor suppressor gene producing Peutz-Jegher's syndrome (Shaw RJ et al., 2004). AMPK was found to activate Tsc2 GAP activity by phosphorylating Tsc2 at two residues in mammalian cells under energy starvation conditions (Inoki K et al, 2003). By integrating and coordinating these cellular energy status stimuli, Tsc2 protects cells from apoptosis induced by glucose limitation. This mechanism points to a promising therapeutic intervention for patients suffering from Peutz-Jegher's syndrome.

f. Hypoxia

Among the environmental cues that govern growth and proliferation, low oxygen levels act as cellular stress to trigger the shutdown of a number of pro-growth biological processes such as translation and gene expression. Hypoxic stress induction of a transcriptional response via activation of the key transcription factor, HIF1, to attenuate energy needing processes has been well studied while translational regulation under hypoxia is not well understood. In addition to HIF1, hypoxia induces two negative regulators of growth in *Drosophila*, *scylla* and *charybdis*, which are the homologs of mammalian REDD1. *scylla* is a direct target of *Drosophila* HIF1 (Reiling JH and Hafen E, 2004). Using genetic interaction studies, it was demonstrated that *scylla* and *charybdis* function downstream to *PKB* but upstream of *Tsc1-2* to alter translational activity of TORC1 but not Akt-mediated FOXO transcription. Similarly in mammals, REDD1/RTP801 inhibits TORC1 signaling towards translation and protein synthesis in a TSC-dependent manner (Brugarolas J et al., 2004). It has been demonstrated that mTOR and its substrate regulation by dephosphorylation under hypoxia is not affected by the decreased ATP levels (Arsham AM et al., 2003), though REDD1 can inhibit mTOR-induced growth under levels of energy stress (Sofer AK et al., 2005). Quite recently, a new mechanism put forth is that hypoxia and REDD1 phosphorylate Tsc2, releasing it from its growth factor stimulated binding with 14-3-3 thus inhibiting mTORC1 activity (De Young MP et al, 2008).

Additionally, Ptp61F (protein tyrosine phosphatase 61F) has been recently indentified in *Drosophila* S2 cells as a novel negative modulator of TOR mediated translational growth in a RNAi screen (Lee SJ et al., 2008). Inactivating Ptp61F prolongs cell survival under hypoxic conditions in a TOR-dependent manner. Thus Ptp61F inhibits TOR-mediated protein synthesis in cells under hypoxia, though the exact mechanism by which it does so is unclear and needs investigation.

g. Phospholipase D and Phosphatidic Acid

The important messenger PA (Phosphatidic Acid) is generated on the hydrolysis of PC (Phosphatidyl Choline) by PLD (Phospholipase D). PLD itself is involved in a number of physiological processes including cytoskeleton organization, membrane trafficking and oncogenic stimulation (Foster DA and Xu L, 2003). Inhibiting PA accumulation by use of chemical inhibitors or via mutagenesis of PLD in mammalian cells, demonstrated that PA is required for activation of mTORC1 translational targets, independently from that mediated by PI3K (Fang Y et al., 2001, Fang Y et al., 2003). This study also identified a competitive interaction between rapamycin and PA for binding mTOR. Using overexpression and RNAi knockdown analysis, it has recently come to light that Rheb is required for mitogenic stimulation of PLD activity (Sun Y et al., 2008). This study found that *in vitro*, Rheb directly bound and activated PLD in a GTP-dependent manner. This implies that Rheb-GTP mediated activation of PLD leads to accumulation of

PA, which can then directly bind to and activate mTOR. The exact mechanism of how PA activates mTOR awaits clarification along with conclusive genetic interaction studies.

h. Amino acid transporters – Pat, Slimfast, minidiscs

The eukaryotic specific amino acid permease transporter family (AAAP) is conserved in all eukaryotes from yeast to metazoans. All mammalian AAAPs known to date share the two common features that they recognize amino acids or similar compounds and their activity depends on the intra and extracellular H+ concentration (reviewed in Boll M et al., 2003). In *Drosophila*, the fat body is analogous to the mammalian liver and in response to growth stimulating signals relays nutritional response via unknown mitogenic factor secretion (Britton JS and Edgar BA, 1998). Thus it is not surprising that this is the site of action of the *Drosophila* AAAPs.

A heterodimeric amino acid transporter encoded by *minidiscs* is important for normal growth and proliferation of the larval imaginal discs (Martin JF et al., 2000). *minidiscs* is expressed in the late larval and pupal fat body and is non cell-autonomously required for imaginal disc proliferation. Under nutrient sufficiency, an unknown minidiscs-dependent factor is secreted by the fat body that allows for imaginal disc proliferation enabling normal metamorphosis and development. The developmental defects exhibited in *minidiscs* mutants, which can be overcome if transplanted in wild type hosts early on, are different than the cell autonomous

growth defects seen in *InR/TOR* mutants. It remains to be addressed which amino acids are specifically transported via *minidiscs* and which downstream signaling pathways are affected.

The cationic amino acid transporter *slimfast* was identified in a genetic screen for growth modifiers (Colombani J et al., 2003). Slimfast acts primarily in the fat body, upstream to TOR, in a cell-autonomous way to regulate growth on nutrient sensing. Loss of *slimfast* mimicked global effects of amino acid deprivation. It was elucidated that TOR acts as a nutrient sensor in the fat body and either inhibiting TOR signal or misexpressing *slimfast* in the wing disc is sufficient to cause effects similar to amino acid deprivation. Thus, Slimfast is required for normal organ and cell growth in flies and it plays a role in regulating arginine import in *Drosophila* S2 cells. Most importantly, the localization of Slimfast to the fat body cell membrane needs TOR activity, and TOR signaling was found to inhibit endocytic degradation of Slimfast (Hennig KM and Neufeld TP, 2006). This adds one more complex interactive relationship in the pathway where a TOR regulator can also be subject to TOR activity, perhaps in order to maintain the fine balance between environmental cues and metabolic homeostasis.

Two genes encoding a third type of proton-assisted transporters (*PAT*) were also identified in a *Drosophila* genetic screen as important for cell-autonomous growth in endocrine and non-endocrine tissues (Goberdhan DC et

al., 2005). Genetic interaction studies demonstrate that *PAT*s phenocopy growth defects of *TOR* in cell growth and cell number. They also promote a high affinity transport of alanine, glycine and proline. The effect of alanine transport regulated by PAT transporters can increase the activity of TOR as measured by S6K-phosphorylation.

Taken together, there are definitive studies identifying TORC1 pathway as the nutrient sensor that causes global modulation of growth on amino acid and growth factor availability through affecting humoral mechanisms. Though the exact amino acid sensor in the pathway is not known, it is important that when identified, it needs to be tested in context with the known three Drosophila amino acid transporters. It is postulated that the above transporters modulate TORC1 activity and this local nutrient sensing mechanism needs to be established further.

V. Downstream targets and effects of TOR activity

With the evolution of multicellularity, the TOR pathway has added on to the ancient amino acid sensing attribute the ability to conduct signals from growth factors and cellular stress in order to achieve a global regulation of growth and proliferation. Due to the complexity of its pleiotropic effects, TOR dysregulation leads to a number of human pathologies on either misexpression or miscommunication via the downstream effects of TOR activity, as described below.

a. Translation and protein synthesis

Contribution of TOR activity towards translation and protein synthesis has perhaps been the most well elucidated downstream function of this master regulator to increase cell mass. In response to nutrient availability, TOR phosphorylates S6K and 4EBP in a rapamycin-dependent manner to promote a global increase in translation.

S6K was initially identified as insulin or interleukin induced target that was sensitive to rapamycin (Chung J et al., 1992). S6K plays a significant role in protein synthesis by phosphorylating 40s Ribosomal protein S6 (RpS6). In mammalian cells, mTOR was shown to phosphorylate S6K at two residues in the hydrophobic domain – Thr389 and Ser 412. S6K phosphorylation at Thr389 is sensitive to rapamycin, induced on Rheb overexpression and amino acid addition but is unaffected by insulin addition (reviewed in Jacinto E & Lorberg A, 2008). A model for translational pre-initiation complex assembly through mTORC1 activation of S6K and 4EBP and subsequent phosphorylation and interaction cascade has been put forth (Holz MK et al., 2005).

In mammalian cells, under normal basal conditions, eIF4E is bound by its inhibitor 4EBP, and S6K1 is associated with eIF3. This eIF3 is also loosely associated with 4EBP-eIF4E complex bound to the 5'mRNA cap. Following a surge of mitogenic activation and/or amino acid availability, mTOR-raptor binds to the eIF3-S6K complex leading to phosphorylation of Thr-389. S6K

phosphorylated at Thr-389 binds to PDK1 to undergo phosphorylation at Thr-229, which activates it fully. The activated S6K disassociates from the eIF3 complex and proceeds to phosphorylate RpS6 and eIF4B. eIF4B is a RNA-binding protein that activates the RNA helicase activity of eIF4A and is required for recruitment of ribosomes to mRNA (Rogers GW et al., 1999). eIF4B phosphorylated at Ser422 is recruited to the eIF3:40s pre-initiation complex, where it also recruits eIF4A. The exact details of how eIF4B and eIF4A function in this complex are unclear. Concurrently while S6K is being phosphorylated at Thr389 by mTOR, mTORC1 comes into proximity with the eIF4E-4EBP that is loosely bound to the eIF3 –S6K complex. Here mTOR phosphorylates 4EBP, freeing eIF4E which then recruits eIF4G to the pre-initiation complex. Thus through a series of phosphorylation events, mTORC1 enables pre-initiation complex assembly to promote translation of mRNAs containing long structured 5' regions under conditions of nutrient and energy sufficiency.

In addition to the above model, it is also known that S6K promotes translation by activating E2F (Elongation Factor 2) via repressing the inhibitory signal from E2Fk (Elongation 2 Factor kinase) through its phosphorylation (Wang X et al., 2001).

Originally, phosphorylation of S6 by S6K was postulated to increase translation of mRNAs containing 5'TOP (tracts of oligopyrimidine). 5'TOP containing mRNAs are thought to encode a subset of translational machinery.

This hypothesis could explain the global increase in translation induced by mTORC1. However, it is now demonstrated that translation of 5'TOP mRNAs does not depend on S6 or S6K phosphorylation (Pende et al., 2004, Ruvinsky I et al., 2005). How mTORC1 controls 5'TOP mRNAs remains to be addressed.

b. Transcription

Studies in yeast show that transcription of a number of nutrient and stress responsive genes is regulated by TORC1 (Loewith R et al., 2002, Jacinto E & Hall MN, 2004). Under nutrient replete conditions, TOR negatively controls transcription of starvation-resistant genes by regulating nuclear localization of nutrient-responsive transcription factors. On availability of good nitrogen sources such as glutamate and ammonia, TOR represses the transcription of genes needed to scavenge poor nitrogen sources in a process known as nitrogen catabolite repression. TOR also inhibits the transcription of general stress responsive genes by cytoplasmic sequestration of general stress responsive factors MSN2 and MSN4.

In addition, there is data that yeast translation control is achieved by TORC1 controlled transcriptional regulation of ribosome biogenesis, an energy consuming process that involves all three nuclear RNA polymerases (Pol I-III). Nutrient limitation or rapamycin treatment leads to overall inhibition of Pol I, Pol II and Pol III by decreasing 35S rRNA transcription, ribosomal protein gene transcription and transcription of 5S rRNA and tRNA respectively (Li H et al.,

2006, Powers T and Walter P, 1999, Schmelzle T et al., 2004). Another way that TOR could regulate transcriptional control of ribosomal protein gene expression is by promoting the maintenance of Esa1 histone acetyltransferase complex at promoters of these genes (Rohde JR & Cardenas M, 2003).

A third way that TOR can regulate transcription of genes involved in ribosome biogenesis is by regulating a URI (unconventional prefoldin) that binds to the small RPB5 subunit present in all three RNA Polymerases via phosphorylation (Gstaiger M et al., 2003). URI-mediated nutrient sensitive ribosomal gene transcription is observed in yeast and mammals. In mammals, TORC1 can control ribosome biogenesis at the level of rDNA transcription via regulation of the transcription factors UBF and TIF1A in a S6K-dependent manner (Hannan KM et al., 2003, Mayer C et al., 2004). Genetic analyses demonstrate that *Drosophila TIF1A* is an important downstream target for cell and organismal growth (Grewal S et al., 2007). This study also provides *in vivo* evidence that TIF1A-stimulated rRNA synthesis controls levels of other ribosomal machinery.

Transcriptional profiling in mammalian lymphocytes has shown that rapamycin treatment and amino acid deprivation upregulates genes involved in nutrient catabolism and energy production while downregulating genes involved in lipid and nucleotide synthesis as well as protein synthesis, folding and turnover (Peng T et al., 2002). Given the central sensor, integrator and transducer role

played by the TOR pathway for coupling nutrient availability to energy consuming growth processes, this observation fits the notion.

c. Cell cycle

It is long known that cells coordinate their mass accumulation and cell cycle on nutrient availability, which is important to establish the correct size of cells, organs and organism (Leevers SJ & McNeill H, 2005). Given the tight regulation achieved by TOR for driving mass accumulation under nutrient replete conditions, it is no surprise that TOR is also involved in assessing cell cycle progression under changing nutrient conditions. In *Drosophila* mitotic and endoreplicative cells, TOR functions to regulate transition from G1 to S phase, consistent with the ability of rapamycin to induce a similar G1 growth arrest in yeast and mammalian cells (Zhang H et al., 2000). In accordance with this, cells with hyperactive TOR activity have a relatively shorter G1 phase and exhibit accelerated entry into S phase (Stocker H et al., 2003, Saucedo LJ et al., 2003, Potter CJ et al., 2001). Interestingly, unlike overexpression of cell cycle regulators, which increase cell division rate and not growth rate, and factors like Cyclin D/CDK4, which increase both cell division and growth rate, overexpression of PI3K/TOR increases growth rate and not cell division rate (reviewed in Neufeld TP, 2003). This explains the increased cell and organ size seen on TOR hyperactivity. Overexpression of Cyclin E or E2F in *Drosophila TOR* mutant cells, whereas overexpression of Cyclin D or E2F versus Cyclin E in rat liver

hepatocytes, could rescue G1 arrest (Nelsen CJ et al., 2003), suggesting that the exact manner of cell proliferation governed by TOR may differ tissue wise and in the model system used. Since Cyclin D promotes G1-S progression, its expression is decreased on rapamycin treatment and has an oncogenic activity in increasing both cell cycle rate, translation and protein synthesis (Nelsen CJ et al, 2003). It is thus an attractive possible direct target of TOR that needs to be validated.

In yeast, rapamycin treatment or TOR depletion results in a metabolic state that shares characteristics of stationary phase (G_0) such as G1 arrest, induction of genes involved in stress response, inhibition of transcription, decreased translation and synthesis of glycogen and trehalose (Jacinto E and Hall MN, 2003). Entry into G_0 on TOR inactivation results from accumulation of Rim-15 protein kinase, a downstream PKA-target, in the nucleus and induction of Rim-15 dependent transcription (Predruzzi I et al., 2003). Entry into G_0 is thus under the convergence of signals from TOR, PKA and Sch9 (yeast PKB homolog) and is mechanistically defined, whereas exit from G_0 is not. It has recently been shown that a vacuolar membrane EGO/GSE complex, consisting of Gtr2, Gtr1, Ego1 and Ego2 is involved in orchestrating exit from rapamycin-induced G_0 state, possibly through induction of microautophagy and by subsequent generation of a pool of vacuolar amino acids (Dubouloz F et al., 2005). In addition, the process of microautophagy, which is the direct cytosolic

sequestration by the vacuolar membrane, can also counteract the membrane influx generated by TOR-induced macroautophagy. The exact mechanism and relationship between the EGO complex and TOR, especially towards microautophagy, awaits dissection.

d. Nutrient import

As mentioned earlier, TOR signaling can modulate nutrient import and utilization in yeast by controlling transcription of the appropriate set of genes.

Yeast express a number of amino acid transporters, called permeases, at their cell surface to ensure a constant supply of amino acids. Specific amino acid permeases are sorted to the surface under nutrient replete conditions while general amino acid permeases (GAPs) are degraded. To establish energy conservation under nutrient limiting conditions, GAP1 is sorted to the cell surface instead of being degraded, a process regulated by the EGO/GSE complex members (Gao M & Kaiser C, 2006). TOR regulates this nutrient responsive sorting of general and specific amino acid permeases. TOR prevents ubiquitination, vacuolar targeting and break down of tyrptophan permease TAT2 by phosphorylating and inactivating NPR1, which normally targets TAT2 for turnover (Beck T et al., 1999).

There is evidence that normally growth factor depletion results in reduction of cell surface glucose and amino acid transporters. Growth factor depletion in IL-3-dependent mammalian cell line expressing constitutively activated Akt

exhibited an increased accumulation of these transporters at the cell surface through a mTORC1-dependent mechanism that is yet unclear (Edinger AL & Thompson CB, 2002). Understanding this ancient amino acid sensing step in the pathway is very important and identifying novel regulators and interactors of TOR using our genetic screening strategy, will definitely further our understanding.

e. Autophagy

Autophagy is an evolutionary conserved, catabolic process that occurs in the absence of nutrients and/or during development of an organism involving bulk delivery and subsequent breakdown of cytosolic components via the lysosomal pathway to garner amino acids (Melendez A & Neufeld TP, 2008). This process involves the enclosure of random cytoplasmic fractions in double membraned vesicles called autophagosomes and their successive delivery to the vacuole/lysosomal for breakdown of the cytosolic components to generate molecular building blocks for normal housekeeping, energetic and biosynthetic functions. Autophagy thus is important for cellular homeostasis of energy and nutrition. The autophagic machinery, comprised of the various ATG (Autophagy related) genes, has been evolutionary conserved in yeast, *Dictyostelium*, *C.elegans*, *Drosophila*, *Arabidopsis* and mammals.

The best-known role of autophagy is pro-survival for generating an internal store of nutrients. In accordance with this, the intracellular amino acid levels within Atg7 deficient yeast cells was observed to be low and limiting for protein

synthesis (Onodera and Ohsumi, 2005). Mice mutant for Atg5, an important player for autophagosome formation, were normal at birth (Kuma A et al., 2004). However, most of them died 1 day after delivery because of their inability to tide over neonatal starvation. Thus, post-birth, autophagy plays a crucial role in providing nutrition and energy to mammals. In *Drosophila*, metamorphosis involves breakdown of larval structures such as salivary gland and larval gut. The energy thus generated is used to sculpt the imaginal discs into respective adult organs and for their growth. This process is triggered by induction of the hormone 20-hydroxyecdysone by PI3K and is associated with high levels of autophagy known as developmental autophagy (Rusten TE et al., 2004).

The protective role of autophagy extends to removal of aggregate proteins such as polyglutamine and polyalanine expansion repeats, huntingtin aggregates, amyloid plaques and microbial pathogens, failure of which leads to neurodegenerative diseases and viral infections (Ravikumar B et al., 2002, Sarkar S & Rubenstein DC, 2008, Itzaki RF et al., 2008, J Orvedahl & Levine B, 2008).

Contrary to its justification as a pro-survival catabolic process, autophagy can also promote death. Beclin1, the mammalian homologue of yeast Atg6, functions as a tumor suppressor promoting survival in addition to functioning as an essential gene for developmental growth in *C.elegans* and mice (Aladzitsy I et al., 2008, Yue Z et al., 2003). A number of dying cell types show presence of

caspase-independent autophagic structures in addition to caspase-dependent autophagic induction (reviewed in Baehrecke E, 2005). An independent and complementary nature between autophagy and apoptosis is thus seen to exist without a clear understanding of the mechanism involved. It is possible that Beclin-1 can function as a link between autophagy and apoptosis, two processes that need to be coordinated. Autophagy also plays an important role for damaged organelle turnover especially in mitochondrial homeostasis since damaged mitochondria were found to accumulate in yeast cells mutant for atg1, atg6, atg8 and atg 12 (Zhang Y et al., 2007).

The role of autophagy in nutrient mediated growth has been long evident. Conditions that lead to autophagy induction such as nutrition limitation by starvation, growth factor depletion and rapamycin treatment also lead to reduction of growth. Tumor suppressors such as PTEN, TSC1-2 and Beclin1 positively regulate autophagy providing an example of the importance of autophagy in cell growth. Studies have established that this regulation of growth control may involve signaling from TOR. Normally under nutrient replete conditions, TOR inhibits autophagy through Atg1 phosphorylation, an early step in the autophagic process (Scott RC et al., 2004). When nutrients and growth factors are missing, TOR inhibition of Atg1-induced autophagy is eliminated, leading to recycling of energy and nutrients. Starvation-induced autophagy and constitutively activated autophagic induction can be overcome by TOR

overexpression. While *Atg1 Drosophila* mutant cells do not display overgrowth under nutrient availability, they cannot limit growth under growth suppressive conditions (Scott RC et al., 2007). In corroboration, *Atg1* overexpression leads to cell death via increased autophagy (Scott RC et al., 2007, Ma J et al., 2007) followed by induction of apoptotic death in *Drosophila* cells.

Inactivation of TOR by use of small molecule enhancers of rapamycin, leads to reduced autophagy and toxicity in Huntington disease models as well as reduced bacterial aggregates in tuberculosis models (Floto AR et al., 2007). Rapamycin also induces the clearance of polyglutamine, polyalanine and tau aggregates in *Drosophila* models (Berger Z et al., 2006).

This points to the importance of TOR-mediated regulation of autophagy in human disease progression and its subsequent targeting for drug development.

f. Metabolism

As discussed above, TOR signaling regulates a number of metabolic processes such as amino acid biosynthesis via transcription, sorting of amino acid and glucose transporters, starvation-induced autophagy and microautophagy. Metabolic disorders such as insulin resistance can arise due to the negative feedback loop between TOR signaling and InR signaling. In addition to these, TOR is also implicated in the process of adipogenesis and fat metabolism (Kim JE and Chen J, 2004).

Genetic screening in *Drosophila* has identified a novel gene called *melted* that modulates both TOR and FOXO activity (Teleman AA et al., 2005). *melted* mutants are leaner, with 40% less fat than control flies and have a defect in fat accumulation. The precise mechanism by which *melted* regulates TOR and FOXO are not clear at present, though it presents us with the important role played by TOR in fat metabolism.

The mechanism behind rapamycin-induced inhibition of adipocyte differentiation (Bell A et al., 2000) has recently been elucidated. mTORC1 regulates activation of transcriptional factor PPAR-γ, which positively regulates transcription of C/EBPα and other adipocyte differentiation genes (Kim JE & Chen J, 2004).

g. Vesicular trafficking and Endocytosis

The regulation of amino acid permease sorting in yeast and synthetic lethality observed with Class C Vps mutants has already been discussed above pointing to the TOR-mediated governing of vesicular trafficking processes. In addition a link is implicated between TOR and preautophagosome complex, which functions in vesicle docking and fusion, protein sorting and is important for autophagy via TOR-regulation of Atg1 and Atg8 (Rohde J et al., 2008, Reggiori F & Klionsky D, 2005).

In a genome wide RNAi screen of human kinases, TOR and S6K were uncovered as regulators of endocytosis (Pelkmans L et al., 2005). TOR and S6K

RNAi knockdown inhibited clathrin-mediated endocytosis and increased caveolae/raft-mediated endocytosis. This is validated by *in vivo* genetic studies in *Drosophila* where clathrin uncoating ATPase *Hsc70-4* was identified as a modifier of TOR overexpression eye phenotype (Hennig KM et al., 2006). The study found endocytosis to be defective and restricted in *TOR* mutants and concluded that TOR reversibly regulated bulk and targeted endocytosis to alter nutrient uptake leading to growth.

Keeping in mind TOR regulation of permease sorting in yeast and its link with endocytosis, there is a possibility that amino acid sensing may occur by promoting endocytic import.

h. Lifespan

Pathways that influence growth and metabolism intersect with lifespan. Given the global effects that TOR pathway exerts for regulation of metabolism and the effects its dysregulation have in human disease, it is not surprising to envision the impact of TOR signaling on lifespan.

In a number of eukaryotic species it is now shown that reducing food intake referred to as dietary restriction (DR) or caloric restriction can enhance longevity (Kapahi P and Zid B, 2004). The observed increase in lifespan is thought to be a result of reallocating organisms' metabolic stores towards somatic maintenance rather than towards growth and reproduction (Holliday R, 1989). Though the exact mechanism behind how this shift occurs is unclear, it

has been observed that pathways modulating growth and metabolism in response to nutrient such as InR and TOR are involved in lifespan extension in *Drosophila* and *C.elegans* (Kapahi P et al., 2004, Vellai T et al., 2003, Jia K et al., 2004). Tissue specific activation of TOR signaling in *Drosophila* by overexpressing constitutively active S6K in fat body or muscle tissue resulted in shortened lifespan while inactivating TOR signaling by overexpressing Tsc2, kinase-inactive S6K or dominant-negative TOR extended longevity (Kapahi P et al., 2004). Interestingly, the lifespan extension seen in the above cases was greater when the flies were reared on nutrient rich food versus nutrient poor food, suggesting a connection between TOR-mediated nutrient sensing and its affect on ageing. TOR deficiency in *C. elegans* caused animals to enter into a developmental arrest in the L3 stage and a continued perseverance in that phase for longer time than the control wildtype animals (Vellai T et al., 2003). A mutation in Raptor (DAF15), a TORC1 component, was also found to extend lifespan (Jia K et al., 2004).

In *Drosophila*, inhibiting insulin signaling by mutating insulin receptor (InR) or the InR adaptor, *chico*, extends lifespan by 50% (Hwangbo DS et al., 2004). InR signaling, which cross talks with TOR pathway has been shown to increase longevity through FOXO. FOXO is a stress-induced transcriptional activator that is inactivated directly on phosphorylation by the TORC2-target Akt and results in its cytoplasmic retention (Puig O et al., 2003). Activation of FOXO in the adult

pericerebral fat body extends lifespan, increases resistance to oxidative stress and reduces lipid metabolism. FOXO activation reduces dilp-2 (*Drosophila* insulin peptide-2) production in neurons and thus by repressing endogenous insulin signaling, provides a non-cell autonomous way to enhance longevity. Thus under nutrient rich condition, FOXO is phosphorylated by Akt and cannot activate its downstream target gene expression. FOXO also cross talks with TOR pathway by transcriptional activation of Raptor (Jia K et al., 2004), 4-EBP and InR on direct binding to their promoters (Puig O et al., 2003) when relieved of Akt-mediated phosphorylation under stress. It has been noted that the resulting lifespan extension on InR mutation requires FOXO activity but FOXO activity is not needed for longevity observed in the *C.elegans* TOR mutant (Vellai T et al., 2003). TOR extension of longevity is therefore independent from FOXO and the exact mechanism needs further elucidation.

Based on the importance of TOR in ageing, any novel regulators or effectors of TOR when identified could provide an insight into this biological process.

i. Neuronal function

The connection between InR signaling and axon guidance in *Drosophila* has been established. The InR itself has been found to be important for axon guidance from the retina to the brain, functioning independently of *chico*, the IRS-like homolog, to act as a guidance receptor for the adapter protein Dock

(*Dreadlocks*) (Song J et al., 2003). Similarly, a very recent study shows the involvement of Tsc1 and Tsc2 in mammalian axon formation and growth (Choi YJ et al., 2008). Overexpression of Tsc1 or Tsc2 suppresses axon formation, while inactivating Tsc1 or Tsc2 induces ectopic axons *in vitro* and in the mouse brain, in part by upregulating SAD kinase which is involved in establishing neuronal polarity. This study is consistent with the defects seen in the *Tsc1* mutant mouse model, which involve enlargement of cortical and hippocampal neurons along with presence of ectopic neurons in the cortex and hippocampus (Meikle L et al., 2007). A postnatal 7-30 day Rapamycin treatment can rescue some of the neuronal defects that are observed in this mouse model, increasing its survival time (Meikele L et al., 2008), suggesting that mTORC1 inactivation can attenuate some of the developmental defects seen in patients suffering from Tuberous Sclerosis.

Most interestingly, another study has observed hyperphosphorylation at the Akt-target site Thr1462 within Tsc2 found in brain cortex samples of patients suffering from Parkinson's and Alzheimer's and in samples from a mouse model of Parkinson's (Habib SL et al., 2008). This study postulates a new mechanism for Parkinson's or Alzheimer's disease, where hyperactive phosphorylation of Tsc2 by Akt may lead to neuronal cell death, giving rise to disease progression.

TOR and InR pathways have been implicated in coordination of neuronal differentiation by regulating the timing of photoreceptor differentiation in

Drosophila eye thus linking it to nutritional and growth factor availability. *Tsc1*^{29} mutant mosaic eyes exhibit both overgrowth and precocious development of photoreceptor cells (Bateman and McNeill, 2004). It is now elucidated that neuronal differentiation and growth diverge downstream of InR/TOR signaling (Mc Neill H et al., 2008). TOR and S6K and not the other TOR target 4EBP, along with eIF4E, mediate the temporal differentiation in the eye. By showing that TOR regulates transcriptional activation of EGFR pathway members, Argos, Rhomboid and Pointed P2, this study provides a mechanistic way for all three pathways, namely InR, TOR and EGFR to interact and control temporal differentiation of photoreceptors in the developing *Drosophila* eye.

TOR signaling via its regulation of translation seems to be important in maintaining protein-synthesis dependent synaptic plasticity. Fragile X Syndrome exhibits one such cognitive malfunctioning due to the absence of FMRP (Fragile X mental retardation protein), a dendritic binding protein that normally represses translation of specific messages. Quite recently it has been observed that mTOR-dependent S6K1 kinase activity following brief PP2A-mediated (Protein Phosphatase 2A) dephosphorylation is important for phosphorylating FMRP and promoting its activity (Narayanan U et al., 2008).

TOR has also been shown to facilitate long-term memory formation in *Aplysia californica*. TOR activation may contribute to lasting synaptic plasticity in

this model system via increasing translation through 4EBP-phosphorylation (Caroll M et al., 2006).

Thus the impact of TOR signaling and its clinical significance for neuronal function has recently come under investigation. Furthering the insight into its mechanistic regulation is important for drug design as it seems to have a conserved function in not only brain malfunctioning but on overall growth and metabolism.

j. Cytoskeletal dynamics

TORC2, the rapamycin-resistant branch of the TOR pathway has emerged as the spatial regulator of growth via regulation of cellular actin dynamics. It was observed that knockdown of mTORC2 in NIH-3T3 fibroblasts inhibited actin polymerization and cell spreading (Jacinto E et al., 2004). Knockdown of Rictor, mTORC2 specific component, lead to formation of abnormal bundles of F-actin intracellularly and modified the localization of cytoskeletal adaptor protein (Sarbassov DD et al., 2004).

Studies in yeast have long identified the importance of TORC2 in actin polarization (Schmidt A et al., 1996). TORC2 regulates actin cytoskeleton through Rho1 mediated activation of PKC-MAPK. TORC2 activates Rho GTPase by activating the Rho-GEF, ROM1 (Schmidt A et al., 1997). The actin depolarization and growth defects seen in *rho1* and t*or2* temperature-sensitive mutants can be rescued by upregulation of Pkc1 and Mapk1, downstream

effectors of PKC signaling (Helliwell SB et al., 1998). Two other downstream effectors of TORC2 important for actin polymerization and cell wall integrity are Ypk2, a yeast AGC kinase orthologous to *GAD8* and a homologous protein pair Slm1-Slm2 kinases (Fadri M et al., 2005). TOR2 directly phosphorylates Ypk2 (Kamada Y et al., 2005). Interestingly, the kinase activity of Ypk2 is dependent upon PKH1 and PKH2, two genes that encode PDK1-like kinase. The exact downstream effects of TORC2-mediated Ypk2-phosphorylation and their connection to Rho1 GTPase are not clear at this point and await elucidation.

Another important link between TORC and cytoskeletal dynamics is through regulation of CLIP170. CLIP170 is a novel plus end microtubule binding phosphoprotein that on phosphorylation controls microtubule dynamics and organization. Biochemical and genetic evidence show that TOR regulates microtubule assembly and function by regulating the yeast homolog of CLIP-190, known as Bik2 (Choi JH et al., 2000). Rapamycin phenocopies the defects in microtubule dynamics, spindle orientation, nuclear positioning and movement seen in *bik2* mutants, suggesting involvement of TORC1 for these processes. TOR binds to mammalian CLIP170 via its N-terminal HEAT repeats and directly phosphorylates it *in vivo* on both rapamycin sensitive and resistant sites (Choi JH et al., 2002). The TOR-mediated phosphorylation of CLIP170 is important for microtubule binding properties of CLIP-170. Not much is known about CLIP170

or how TOR regulation affects CLIP170 binding to other proteins that exist at the kinetochore.

Dictyostelium is an attractive model to study chemotaxis, a process that is important not only for organismal development during embryogenesis, in inflammation and wound healing but also in malignant cells for metastasis. In order to form a multicellular body, unicellular haploid *Dictyostelium* cells need to undergo accurate chemotaxis as well as relay of the cAMP signal, which activates adenyl cyclase via G-protein signaling. Though it has been long thought that PIP3 is important for migration in *Dictyostelium*, neutrophils and amoeba, knockdown of all five *Dictyostelium* PI3-kinases showed that polarization of PIP3 at the membrane is not necessary for accuracy of chemotaxis but is more important for normal cell speed (Hoeller O & Kay RR et al., 2007). This opened up the stage for involvement of the TORC2 signaling pathway for chemotaxis. Mutations in the *Dictyostelium* TORC2 complex lead to inhibiting multicellularity by exhibiting reduced cell polarity, decreased chemotaxis speed and directionality, reduced phosphorylation of Akt and decreased adenyl cyclase activation (Lee S et al., 2005). Accordingly this PIP3-independent pathway important for chemotaxis was identified as TORC2 using genetic and biochemical studies (Kamimura Y et al., 2008). It was found that a unique constitutively active membrane bound form for Akt (called PKBR1), that lacks a PH domain and is myristylated, is activated at the cell leading edge by chemoattractant induced

heterotrimeric G-protein signaling. This PKBR1 then goes on to phosphorylate other targets. The formation of activated PKBR1 is dependent upon phosphorylation by TORC2 and is induced at the leading edge by cytosolic TORC2. This spatial regulation by TORC2 is necessary for accuracy of chemotaxis and places TORC2 at the junction of another biologically important process.

Due to the recent identification of TORC2 complex, there are a number of unidentified substrates and functions for it. Identifying the regulators, effectors and interactors of TORC2 by using classical genetic screen, opting for high-throughput genomic screens or proteomics approach, will help us understand the significance of TOR-mediated spatial control of growth.

k. Ceramide Biosynthesis

Slm1 and Slm2 two PH containing proteins were identified as novel binding partners of PI-4,5-P_2 (Fadri M et al., 2005) and get directly phosphorylated by TORC2 to regulate actin organization and cell wall integrity. In yeast, calcineurin, a conserved Ca2+/calmodulin dependent protein phosphatase is activated on stress to promote cell survival by induction of a stress-response set of genes (Cyert MS, 2001). TORC2/SLM and calcineurin signaling were found to be mutually antagonistic (Mulet JM et al., 2006). Yeast cells mutant for TORC2 or TORC2 effectors SLM1 and SLM2 exhibited increased activation of calcineurin-dependent transcription and actin depolarization. Cells mutant for the

calcineurin docking site on SLM1 were highly sensitive to oxidative stress while this mutant Slm1 protein was insensitive to calcineurin. Similarly, calcineurin defective cells demonstrated Slm1 hyperphosphorylation and exhibited strong interaction with TORC2. In an independent study, using synthetic gene array analysis, it was shown that Slm1 and Slm2 regulate actin cytoskeleton organization due to their role in sphingolipid metabolism via directly binding to calcineurin and its consequent negative regulation (Tabuchi M et al, 2006). The actin cytoskeletal defects seen in SLM1 and SLM2 could be suppressed by alterations in inositol phosphoceramide (IPC) metabolism via calcineurin inactivation and/or by loss of Isc1, the yeast inositol phosphosphingolipid phospholipase C that was identified as a Slm1/2 target. This points to the ability of SLM1 and SLM2 to cross talk between phosphoinositide, ceramide and TORC2 pathways.

Another study furthered the ceramide-TOR connection recently. TORC2 was found to regulate ceramide synthesis via regulation of ceramide synthase (Aronova et al., 2008). Ypk2, a target of TORC2, and avo3 (Rictor) temperature-sensitive mutants share similar defects in cell wall integrity and actin polymerization. Ypk2 was hypophosphorylated in avo3 mutants and overexpression of a constitutively active Ypk2 can overcome the actin polymerization defects seen in avo3 cells. Ypk2 upstream regulators PKH1 and PKH2 are also directly activated by the sphingoid base phytosphingosine (Friant

S et al., 2001). Aberrant ceramide synthesis was observed in TORC2 mutants *in vitro* and *in vivo*. In an extension of studies by Kamada et al. and Mulet et al., it was found that TORC2 regulates ceramide biosynthesis in part by phosphorylating Ypk2. Additional studies are needed to determine the exact relationship and distinction between the TORC2-SLM and TORC2-YPK2 regulation of ceramide biosynthesis.

At this point it is not clear if the role of TORC2 regulation of ceramide biosynthesis will be conserved in higher eukaryotes. In mammals, ceramides, sphingosine and sphingosine-1-phosphate, which play a role in survival and proliferation, are produced by recycling rather than biosynthesis. Ceramides in higher eukaryotes such as mammals and *Drosophila* are attributed with stress induced growth inhibition and apoptosis promotion (Obeid LM & Hannun YA, 2003). Nevertheless, analysis of ceramide and its breakdown product, sphingosine, in mammals can unveil new roles for TORC2 signaling.

VI. Regulatory feed back mechanisms

Recent studies have emphasized an existing negative feed back loop in the TOR pathway where some TOR targets effectively act as its upstream regulators. This feed back loop acts between the TSC-mTOR-S6K pathway and IRS1-PI3K-Akt pathway (Shah OJ et al., 2004, Harrington LS et al., 2004). These studies show that constitutively activated S6K attenuates insulin signaling by repressing IRS1 gene expression and by directly phosphorylating it. This

supports the observation that both S6K knockout and phosphorylation-deficient S6 mice exhibit insulin sensitivity but are not hypoglycemic (Um SH et al., 2004, Ruvinsky I et al., 2005). This suggests that not only S6K but also S6 is somehow involved in the negative feedback loop. This finding is important in a clinical setting as it explains the development of metabolic syndromes such as diabetes and obesity. Two groups have also independently shown that S6K, the most commonly used readout for TORC1 activity, phosphorylates mTOR at Ser2448 and Thr2446 (Holz MK et al., 2005, Chiang GG and Abraham RT, 2005). As of now, the significance of these phosphorylation events, their nature and which TORC is affected by it are all unknown and wait investigation.

It has been revealed that while Akt activates TORC1, TORC2 phosphorylation of Akt at Ser473 within the HM fully enhances Akt-stimulation by PDK1 (Sarbassov DD et al., 2005). TORC2-mediated phosphorylation of Akt is critical for its complete activation and is needed for downstream effects of Akt.

Thus, we can infer that with the evolution of multicellularity the ancient nutrient sensing TOR pathway interplays with and integrates growth factor signals to ensure that the metabolic processes of the cell and organism do not work for conflicting purposes.

VII. Localization of TOR

TOR kinase exerts the above-mentioned downstream functions through a combination of its kinase activity, association with regulatory factors, or by

accessing its substrates directly by subcellular proximity. It is unclear how assembly of the two different TORCs is signaled. It is therefore possible that subcellular localization of the different TORC components, its upstream regulatory components and downstream effectors, play an important physiological role in transducing TOR signaling effects.

Studies using subcellular fractionation in 3T3 adipocytes and immunoflouresence in yeast have shown that TOR associates with plasma membranes, Golgi and endoplasmic reticulum (ER) and a non-secretory or non-endocytic vesicular compartment (Withers DJ et al., 1997, Kunz J et al., 2000, Drenan RM et al., 2004). Two distinct amino acid regions within the N-terminal HEAT repeats mediated the localization of Tor2 to the plasma membrane, implicating the importance of protein-protein interactions in yeast cells (Kunz J et al., 2000). In line with TOR acting as a cellular nutrient sensor, it is not surprising that TOR localizes to ER and Golgi, which are sites important for nutrient sensing and signaling (Drenan RM et al., 2004)

A separate study in yeast found that Tor1, Tor2, LST8 (GβL) and Avo3 (Rictor) associated to intracellular membranes, possibly endosomal, that were distinct from the plasma membrane (Wedaman KP et al., 2003). This is supported by a number of regulatory and downstream effects of TOR signaling in endocytosis and sphingolipid metabolism. Interaction between Tor2 and LST8

could be stabilized on its association with intracellular membranes, suggesting importance of localization for TORC2 function (Wedaman KP et al., 2003).

In yeast, Tor1 possesses a NLS (Nuclear Localization Sequence) near its kinase domain and NES (Nuclear Export Sequence) and is dynamically distributed in the cytoplasm and nucleus in a rapamycin and nutrient sensitive manner. (Li H et al., 2006). Normally Tor1 resides in the nucleus where it influences growth and transcription by directly associating with the 35rDNA promoter region. On nutrient limitation or rapamycin treatment, it is exported out via an exportin. DNA binding abilities are not required for the nuclear retention TOR1 (TORC1) and thus there still exists some cytosolic TOR for translational upregulation.

In lieu of its critical importance in translation and protein synthesis, mTOR is predominantly cytoplasmic. However, recently the nucleo-cytoplasmic movement of mTOR is shown to be important for maximal activation of S6K1 (Bachmann RA et al., 2006). Plasma membrane localization of upstream regulators of TOR such as Akt, TSC and Rheb is also important for their activity. Akt-phosphorylation of Tsc2 represses its activity by translocating it to the cytoplasm, thus sequestering it away from its direct target, Rheb (Cai CL et al., 2006). Rheb gets farnesylated and localizes to the endosomal membrane instead of the plasma membrane due to absence of palmitoylation (Takahashi KM et al.,

2005). Localization to the endomembrane is critical for Rheb-mediated TORC1 activation following GTP binding of Rheb (Buerger C et al., 2006).

Recent studies in yeast and mammalian cells have implicated components of vesicular trafficking, such as those of the Class C Vps complex, as regulators of TOR activity in response to amino acids (Zurita-Martinez et al., 2007, Nobukini T et al., 2005). These observations again support localization of TOR on endosomal vesicular surfaces and suggest the importance of influence of subcellular localization for TOR regulation and function.

Most recent studies (Sancak Y et al., 2008) present a model where cytoplasmic TORC1 gets activated on amino-acid sensing and translocates to a Rab7-containing vesicular membrane, where it gets directly activated by Rheb for stimulating protein synthesis and translation. This mechanism will be discussed in Chapter 5. This again agrees with the notion of TOR and Rheb localization at the endosomal membranous structures for achieving downstream TORC1 functions.

VIII. Summary

Due to its master regulatory nature for achieving growth and proliferation in response to environmental stimuli of nutrients, energy and stress, the TOR pathway is connected to a network of diverse growth-regulating signals and pathways. Owing to its pleiotropic downstream effects in growth and development, dysregulation of TOR signaling has been implicated in human

pathologies such as cancer, cardiac hypertrophy, metabolic syndromes such as diabetes and obesity, and neuronal and ageing related-disorders such as Alzheimer's disease. In humans, mutations in genes involved in the TOR pathway or its regulation such as *TSC1*, *TSC2*, *LKB1*, *VHL*, *NF1* and *PKD1* result in genetic disorders like Tuberous Sclerosis, Peutz-Jegher's syndrome, Cowden syndrome, von Hippel-Lindau disease, Neurofibromatosis Type 1 and Polycystic Kidney disease, respectively (reviewed in Rosner M et al., 2008). Currently, rapamycin and its analogs are in phase III clinical trials for drugs for Tuberous Sclerosis and breast cancer. These compounds are already in use as immunosuppressants and against renal carcinomas (Llovet JM et al., 2008). Due to hyperactive TOR signaling observed in many cancers, development of TOR and its various effectors as prognostic markers can also be a significant step for cancer progression and therapy response prediction in cancer patients (Hwang M et al., 2008).

In order to use rapamycin or its analogs efficiently in clinical treatment, it is crucial to gain a detailed understanding of the different signaling pathways it perturbs. Identification and characterization of novel regulators, substrates and macromolecular binding partners of TOR complex is therefore extremely crucial for furthering the diagnosis, understanding and treatment of human pathologies arising from aberrant TOR signaling.

There exist a number of unanswered questions in the field of TOR signalling. While new regulatory connections to TOR pathway are coming to light, one of the most ancient aspects of TOR pathway, the exact nature of amino acid sensing, has remained elusive. How amino acids are sensed and how amino acid regulation of TOR fits into the recently emerged concept of temporal (TORC1) versus spatial (TORC2) growth achieved by this pathway, are key questions. In response to these unanswered questions we set our goals to identify novel positive regulators and effectors of TOR signaling drawing on the power of reverse and forward genetic approaches in *Drosophila*, a historically studied and genetically amenable model organism, the results of which are presented in chapters 3 and 4.

In *Drosophila* life cycle, the animal exists in the larval stage for about 4 days where it undergoes continuous feeding and later during the pupal stage relocates these nutrients via breakdown of stored fats. Thus the larval stage is a good point to analyze the effects of nutrient-dependent regulation of growth and metabolism. The larval fat body plays an important role in sensing nutrients and relaying nutritional response via humoral mechanisms, thus affecting overall organismal metabolic response (Colombani J et al., 2003). It is therefore a good tissue to study regulation of and by TOR signaling.

Studies presented in this thesis identify dRag-GTPases as critical for amino acid mediated regulation of TORC1. This study also presents a possible

mechanism for dRag-mediated TORC1 activation through localization studies of FLAG-tagged TOR. dRag activates TOR by localizing it to the cytosol and cell membrane. Our studies indicate that another novel player, Arf79F, the fly homolog of Arf1, mediates this translocation of TOR on dRag activation. Arf79F is required for dRag activation of TORC1 as seen with immunohistochemical studies. Loss of Arf79F inhibits TORC1-mediated growth and cell proliferation. Our studies have thus been successful in answering some of the long-standing questions and furthering the understanding of the TOR pathway and its regulation of growth.

CHAPTER 2: MATERIALS & METHODS

I. Mutagenesis:

3-5 day old males (yw; Py^{+}; $Tsc1^{29}$,w^{+}/TM6BTb) were placed in empty bottles for 30 mins. for dehydration at room temperature. 3 mL of 25 mM EMS solution (26.5μL EMS in 10 ml 100mM Tris pH 7.5 and 10% sucrose) was used to saturate filter papers placed at the bottom of an empty vial. The dehydrated males were placed in this vial containing the mutagen overnight. Next morning the males were transferred to fresh empty tubes to allow them to discard extra EMS and prevent toxicity. After 30 mins. the mutagenized males were placed on fresh food to allow recuperation and after a few hours were crossed to the female parents (yw; isogenised 2^{nd} chromosome; $Tsc1^{29}$/TM6BTb) in a 1:1 ratio. The crosses were flipped for four days and discarded on the fifth. All crosses from F2 onwards were set up with one individual male. A ratio of 1 male to 3 females was maintained for all crosses in vials to keep crowding conditions and food availability uniform. All crosses were maintained on regular fly food (cornmeal, molasses and agar) at 25ºC.

II. Mapping:

Deficiency Mapping: Deficiency mapping was carried out using Exelesis second chromosome Deficiency kit that covers about 56% of the genome with an average deletion size of about 140 kb. Exelesis third chromosome kit and available lines from Drosdel third chromosome (courtesy Thomas Hays' lab) were

used for deficiency mapping of the third chromosome suppressors. The average deletion size of Drosdel deficiencies is about 467 kb and the kit covers about 50% of the entire genome. Together both these kits provided 90.2% coverage of 3L and 80.6% coverage of 3R. Males from the deficiency kits were crossed to the appropriate suppressor bearing females in a ratio of 1:3 and were reared at 25ºC.

Molecularly defined P-element insertion mapping: 12 P-element insertional lines that were 10 map units apart on the second chromosome, with the same genetic background and carrying the w^+ visible marker were purchased from the Bloomington Stock Center. Each P-line was crossed appropriately to create stocks of yw; P{w+}/cyo; Sb/TM6BTb. These males were crossed to yw; M2/cyo; $Tsc1^{29}$/TM6BTb to obtain females yw; M2/P{w+}; $Tsc1^{29}$/TM6BTb where spontaneous recombination could occur. Post recombination, the number of recombinant progeny was used to determine the distance between each P-insertion line and M2. The P-element insertional lines used were as follows - y^1 $w^{67}c^{23}$; P{SUPor-P}KG00569 (at 21B1), y^1 $w^{67}c^{23}$; P{SUPor-P}msl-2KG06747 (at 23F3), y^1 $w^{67}c^{23}$; P{SUPor-P}KG07822 (at 26D7), y^1 $w^{67}c^{23}$; P{SUPor-P}KG01020 (at 28E7), y^1 $w^{67}c^{23}$; P{SUPor-P}KG01121 (at 31D7), y^1 $w^{67}c^{23}$; P{SUPor-P}Ance-3KG04754 (at 34E4), y^1 $w^{67}c^{23}$; P{SUPor-P}KG01626 (at 45A1), y^1 $w^{67}c^{23}$; P{SUPor-P}KG04646 (at 50F1), y^1 $w^{67}c^{23}$; P{SUPor-P}KG00955 (at 53C3), y^1 $w^{67}c^{23}$; P{SUPor-P}hrgKG01510 (at 56E1), y^1 $w^{67}c^{23}$; P{SUPor-P}KG02493 (at 58E1). All fly crosses were reared on standard fly food at 25ºC.

III. Drosophila Stocks and genetic manipulations:

ESTs encoding dRagA (CG11968) and dRagC (CG8707) were purchased from Drosophila Genomics Resources Center (Bloomington, IN). Wild type and PCR-generated amino acid substituted mutants were created (by E.Kim), sub-cloned into pUAST and injected into w*118* embryos using standard protocol (Model System Genomic, Duke University). dRag-transgenic overexpression was achieved by GAL4-UAS system as described below. P[EPgy2]EY11726 a lethal P-insertional mutant in the 5' UTR of CG8707 (dRagC) was used to analyze endogenous disruption of dRagC. Lethality of this line, henceforth referred to as dRagC, was reversible on mobilization of the P-element. dRagC was recombined with FRT42D to generate homozygous loss of function dRagC clones as described below.

UAs-Arf79FRNAi transgenic flies were purchased from Vienna Stock Center.

Flies were reared on standard food with cornmeal, molasses and agar at 25°C, unless noted otherwise.

IV. Overexpression and loss-of-function clonal analysis in Drosophila:

Gain-of-function clones:

Act>CD2>GAL4,UAS-GFP flies were mated with flies carrying hsflp,UAS-gene of interest. Spontaneous FLP-mediated recombination results in excision of the CD2 cassette thereby allowing Act-induced GFP and UAS-dRag clonal expression. For Atg8-GFP expression, Act>CD2>GAL4,UAS-GFPAtg8a flies were used as above allowing Act-induced UAS-GFPAtg8a and UAS-dRag clonal expression. This technique was used to create fat body clones with dRagAWT, dRagAQ61L (constitutive active), dRagAT16N (dominant negative), dRagCWT, dRagCQ99L (constitutive active), dRagCS45N (dominant negative), Rheb, and Arf79F-RNAi expression in *Drosophila* fat body. Fat body samples from Act>CD2>GAL4,UAS-GFP flies crossed to plain hsflp were used as control.

Loss-of-function clones:

hsflp induced FRT-mediated clones were created using dRagC,FRT42D and $Rheb^{2d.1}$,FRT40D in *Drosophila* fat body. FLP was induced in 0-8 hour embryos via 2-hour long heat shock in an air incubator at 37°C. Fat body driver cgGAL4 was used to drive UAS-GFP lines on the appropriate FRT-bearing chromosomes

for uniform GFP expression. Loss-of-function clones were marked by the absence of GFP expression.

eyflp induced FRT-mediated clones were created in the eye using $Tsc1^{29}$, FRT82B by crossing to a FRT82B chromosome carrying the w^+ cell lethal marker. On induction wildtype eye cells, if alive, were marked with w^+. Genotype of imaged flies was eyflp/yw; FRT82B, M,$Tsc1^{29}w^+$/FRT82B,w^+Cell lethal (for third chromosome suppressors and eyflp/yw; M/+ ; FRT82B,$TSc1^{29}$/FRT82B,w^+cell lethal. N≥5 where N=number of adults analyzed.

V. Histology

24 hours prior to histological analysis, larvae with genotype of interest were transferred to fresh food vial with fresh yeast paste. Gentle agitation was used throughout fixing and staining procedures to attain uniform exposure of agents to animal tissue.

Fixation – Third instar larvae were dissected in PBS, cut, inverted and fixed in a solution of 3.7% formaldehyde in PBS for 1 hour at room temperature or overnight at 4°C. Fixative was washed 4 times with PBST (PBS+0.1% TritonX) for 20 mins. each.

FLAG-TOR staining – Animal fat body tissues were tested in either well-fed state or starved for 4-hours on 20% sucrose. Fixed larval samples were blocked in PBSTG (PBST+5% goat serum) for 2 hours at room temperature or overnight at

4°C. Post-blocking, samples were incubated in Anti-FLAG M5 (1:500 Sigma Aldrich) in PBSTG for 2 hours at room temperature or overnight at 4°C, followed by washes with PBST 4X20 mins.). Following a second blocking step as before, samples were incubated in secondary antibody (goat anti mouse 1:500)+1μM Hoechst 33258 for 2 hours at room temperature or overnight at 4°C. After washes with PBS 4X20 mins., samples was dissected in PBS, mounted in Fluoroguard (Bio-Rad Laboratories) and imaged using a 63X objective lens on the CARV.

Phalloidin staining – Animals were tested as either well-fed or starved for 48-hours on 20% sucrose. Fixed larval samples were blocked in PBSTG (PBST+5% goat serum) for 2 hours at room temperature or overnight at 4°C. Post blocking, samples were incubated in 0.165 μM Alexa Fluor Phalloidin (Invitrogen) + 1μM Hoechst 33258 in PBSTG for 2 hours at room temperature or overnight at 4°C, followed by washes with PBS 4X20 mins. Samples were dissected in PBS, fat body mounted in Fluoroguard and imaged using a 40X objective lens. Average area of mutant and surrounding control fat body cells was determined using the histogram function of Adobe Photoshop 7.0.

LysoTracker Staining and assay – Animal fat body tissues were tested as unfixed samples from larvae in either well-fed state or starved for 4-hours on 20% sucrose. Larvae were dissected in PBS two at a time, incubated in 0.1mM

LysoTracker solution + 1μM Hoechst 33258 for 3 mins, rinsed with and mounted in PBS followed by immediate live imaging using a 40X objective lens.

Atg8-GFP assay- Animal fat body tissues were tested as fixed samples from third instar larvae in either well-fed state or starved for 4-hours on 20% sucrose. Samples were imaged using a 40X objective lens and number of GFP-positive punctae was counted.

Imaging and size quantification: Images were captured using ACT1 software to run a DXM 1200 Nikon digital camera attached to a Zeiss Axioscope 2 epifluorescence microscope with a Plan-Neoflar 5X, 40X and 63X objective lens.

All eye images were captured using a Coolpix 990 Nikon digital camera attached to a dissecting microscope.

Post-immunohistochemistry with FLAG- TOR, images were captured by an ORCA-ER, Hamamatsu digital camera on a Zeiss Axioplan 2 microscope equipped with a spinning disc confocal system (CARV; BD Biosciences) with a Plan-Neoflar 63X objective lens.

VI. Wing compartmental and cell size analysis:

Wings from well-fed and starved male adults expressing the indicated UAS-dRag transgenes using dorsal driver *ap-GAL4* and posterior wing driver *en-GAL4* were imaged and analyzed for growth effects. Starvation was achieved by transferring early third instar larvae (72 hour old) to 20% sucrose until pupation followed by

transferring the pupae on food. To quantify transgene effects on adult wing cell and compartment size, the average area and number of cells within a representative anterior region (area between wing margin and L1) and a representative posterior region (area between L3 and L4) was determined using Photoshop 7.0.

VII. Viability of adult flies on starvation

50 female adults expressing the indicated UAS-dRag transgenes using ubiquitous *da-GAL4* or fat body specific *cg-GAL4* drivers were fed on regular food for 24-48 hours post eclosion before complete starvation on plain agar. The survival rates were accounted for on plain agar, with viable flies counted and transferred to fresh agar vials every 24 hours. These experiments were carried out in triplicate and at room temperature.

CHAPTER 3: A FORWARD GENETICS APPROACH TO INDENTIFY NOVEL POSITIVE REGULATORS OF THE TOR PATHWAY

I. A need to identify novel components of the TOR pathway

TOR pathway acts as a global regulator of growth and proliferation on nutrient and growth factor availability in the absence of stress. TOR achieves this coupling of temporal and spatial growth to nutritional availability via involvement with its interactors and binding partners, and subsequent functioning as either a TORC1 or a TORC2 complex. Much that we know about TOR pathway today is still unclear despite the entry of rapamycin in Clinical Phase II trials as a candidate drug for different cancers and solid tumors [Fig3A]. To better our understanding of this key pathway, there is a need to identify novel downstream effectors and novel upstream substrates of TOR action in order to gain a complete picture of the binding partners and cofactors for the different TORCs. Absence of TOR activity results in reduced growth, delayed development and eventually organismal death (Zhang H et al., 2002). Surprisingly, overexpression of TOR in different *Drosophila* tissue results in tissue undergrowth. This can be explained by the increased cell death of TOR expressing cells that is observed in these tissues or it is possible due to titration of TOR complex members by the high amount of TOR protein present (Hennig KM and Neufeld TP, 2002).

TOR hyperactivity in humans results in a number of overgrowth diseases such as Tuberous Sclerosis and cancers. The products of two tumor suppressor genes, *Tsc1* and *Tsc2*, make up the TSC complex, which acts as a negative

regulator of TOR pathway, by the GTPase activating protein (GAP) function exhibited by Tsc2 towards the small G-protein Rheb (Gao X & Pan DJ, 2001). A mutation in either *Tsc1* or *Tsc2* results in a dominant genetic human disorder called Tuberous Sclerosis that occurs at a frequency of 1 in 6000 live births (Rosner M et al., 2008) and is accompanied by symptoms of epilepsy, developmental delay, neuronal pathologies and the presence of benign tumors called hamartomas in different organs and tissues. These hamartomas are also present in other overgrowth disorders such as Peutz-Jegher's syndrome and Polycystic Kidney disease (Rosner M et al., 2008).

In order to answer a number of existing questions in the pathway [Fig 3A] and to gain an insight into new interactions of the TORC components and TOR pathway players, we decided to use forward and reverse genetic approaches to identify novel interactors and regulators in the pathway. We designed an EMS mutagenesis screen as an unbiased way to identify novel positive regulators of the pathway. In a second strategy, we employed a reverse candidate approach, based on literature and research analysis for interesting candidate genes that could interact with TOR pathway members to regulate its activity.

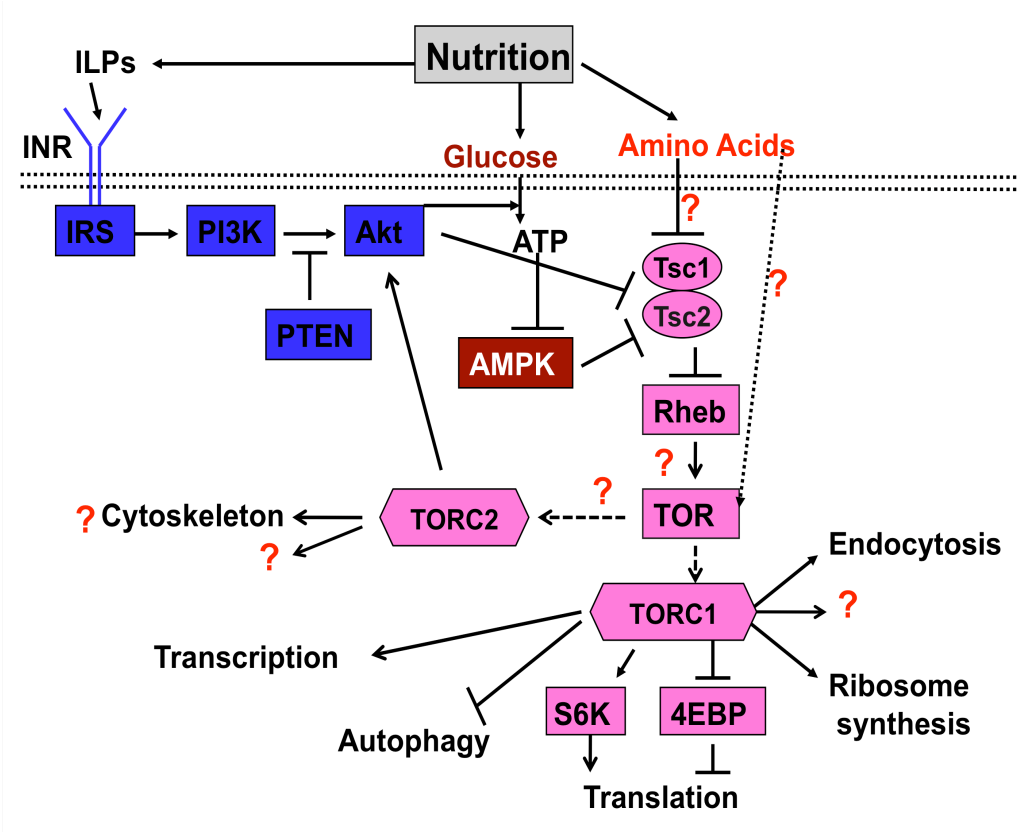


Fig 3A: A brief schematic of the TOR pathway with respect to nutritional regulation

The TOR pathway is chiefly upregulated by the presence of nutrients at three levels by growth factor availability (insulin), amino acid sufficiency and high glucose levels which in turn increase the cellular energy levels. The unknown mechanistic regulation and regulators/effectors for either TOR complex are depicted by question marks. The nature of Rheb-TOR interaction is still unclear though the popular view is that Rheb activates TOR by direct binding. One of the most discussed issues in the field is, how exactly are amino acids sensed and what are the regulators and mechanisms involved for the same. The signal for TOR to recruit binding partners to form TORC1 versus TORC2 and its mechanism is also not known. Though it is known that TORC2 mediates actin cytoskeleton organization, the exact mechanism of this regulation as well as the players involved remain to be unveiled. Due to its pleiotropic nature in controlling growth and proliferation both spatially and temporally, there are a number of unknown downstream effectors of TORC2 and TORC1.

II. Screen rationale

In *Drosophila*, animals that are homozygous null mutants for *Tsc1*^{29} die at early stages of development due to TOR hyperactivity (Radimerski T et al., 2002). The resulting death in these animals can be partially rescued by downregulating the TOR pathway. Removing a single copy of downstream positive players such as *Rheb*, *TOR* or *S6K* can thus partially rescue this early larval lethality to the pupal stage (Radimerski T et al., 2002) [Fig 3B]. We utilized these genetic interactions within the TOR pathway to design a forward genetics approach for identification of novel positive players in the TOR pathway. A chemical mutagenesis screen was designed to identify novel positive players as dominant partial suppressors of *Tsc1*^{29} early larval lethality. The screen would enable us to identify novel players in the pathway that acted downstream or in parallel with TSC and positively regulated TOR activity.

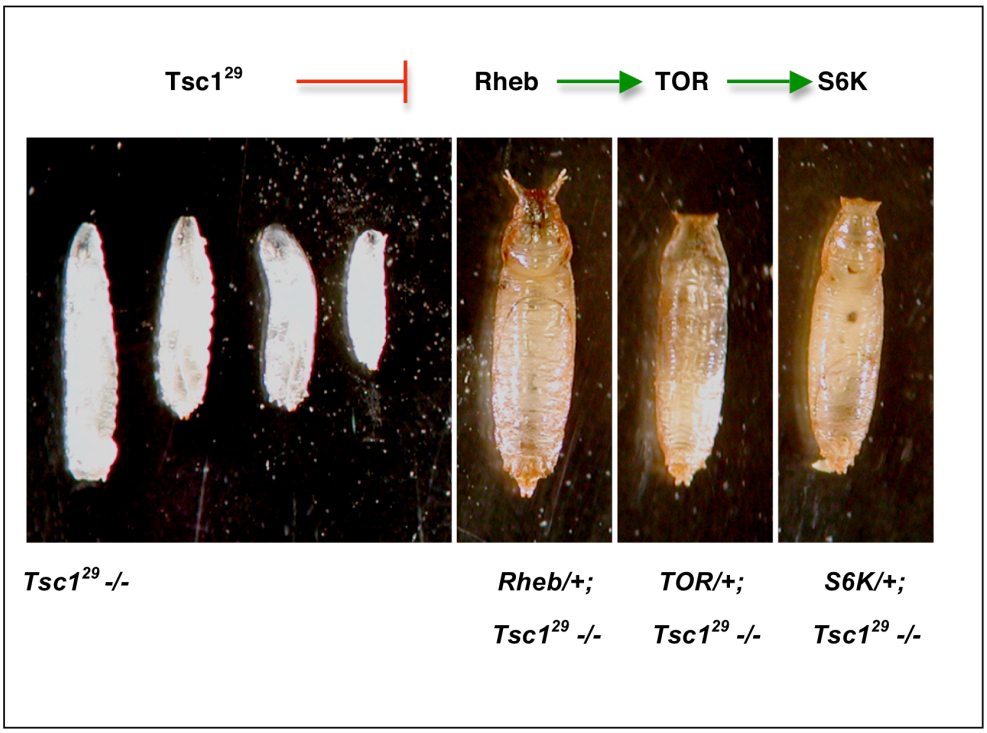


Figure 3B: Dosage sensitive genetic interactions in the TOR pathway.

All *Tsc1*^{29} homozygous mutant animals die as larvae due to hyperactive TOR signaling, based on the amount of nutrition available. Higher amount of nutritional availability results in early death while lesser nutrient availability results in death at early third instar stage. Reducing the downstream TOR signal by removing a copy of any downstream positive player in the pathway such as *Rheb*, *TOR* or *S6K* can partially rescue this lethality to the pupal stage in a dominant manner. These dosage sensitive interactions were used as a rationale for designing a forward genetics screen to identify novel suppressors of *Tsc1*^{29} lethality.

(Images used for generating this figure were taken by former graduate student K.M.Hennig)

III. Screen design

EMS was chosen as a mutagen due to ease of administration and the high rate of mutations that it is known to yield in an unbiased manner. Since the F1 progeny of the mutagenized males is mosaic due to the generation of point mutations on one DNA strand of the mutagenized male, it is possible that the mutation may not be inherited unless the germ cells are mutant (reviewed in St. Johnston D, 2002). To overcome this problem, the screen was carried out at a F2 level to confirm the inheritance of the suppressor of Tsc1 lethality. EMS screens have the disadvantage of laborious and difficult mapping procedures of single point mutations to their respective genes. However, availability of SNP maps of about 50kb regions allows using meiotic recombination mapping, especially for mutants within the same complementation group. EMS generated mutations are unmarked and often times more than one mutation, including lethal mutations, can be induced on a single chromosome complicating mapping and analysis. However, since EMS screens allow for recovery of multiple alleles of a single complementation group (Homyk T et al., 1986), we expected to find a multi allelic complementation group that would enable the cleaning up of secondary lethal mutations by putting two alleles in trans with each other.

An isogenised stock with the second chromosome marked by yellow body color and the third chromosome marked with the *Tsc1*^{29} null allele and red (w^+) eye color was used as the male parental stock for mutagenesis. Males from this

stock were mutagenized using a standard EMS mutagenesis protocol at a concentration of 25mM, where the average rate of mutation for an average gene is ~ 1 in 1000 (Greenspan RJ, Fly pushing). The mutagenized males were crossed to the female parental stock with the third chromosome marked with just the *Tsc1*^{29} null allele. All F1 males were collected and individually crossed to the female parental stock again. At the F2 level, the potentially suppressor carrying second and third chromosome were segregated. The F3 progeny of each individual F2 male carrying either the marked, mutagenized second chromosome or the marked, mutagenized third chromosome was tested for the ability to suppress *Tsc1*^{29} early larval lethality [Fig 3C].

If a suppressor was present, the original individual male parent was used to set up an isogenised stock. To determine the novelty of the suppressor, complementation crosses were carried out with *TOR* for the second chromosome suppressors and *Rheb* and *S6K* for the third chromosome suppressors.

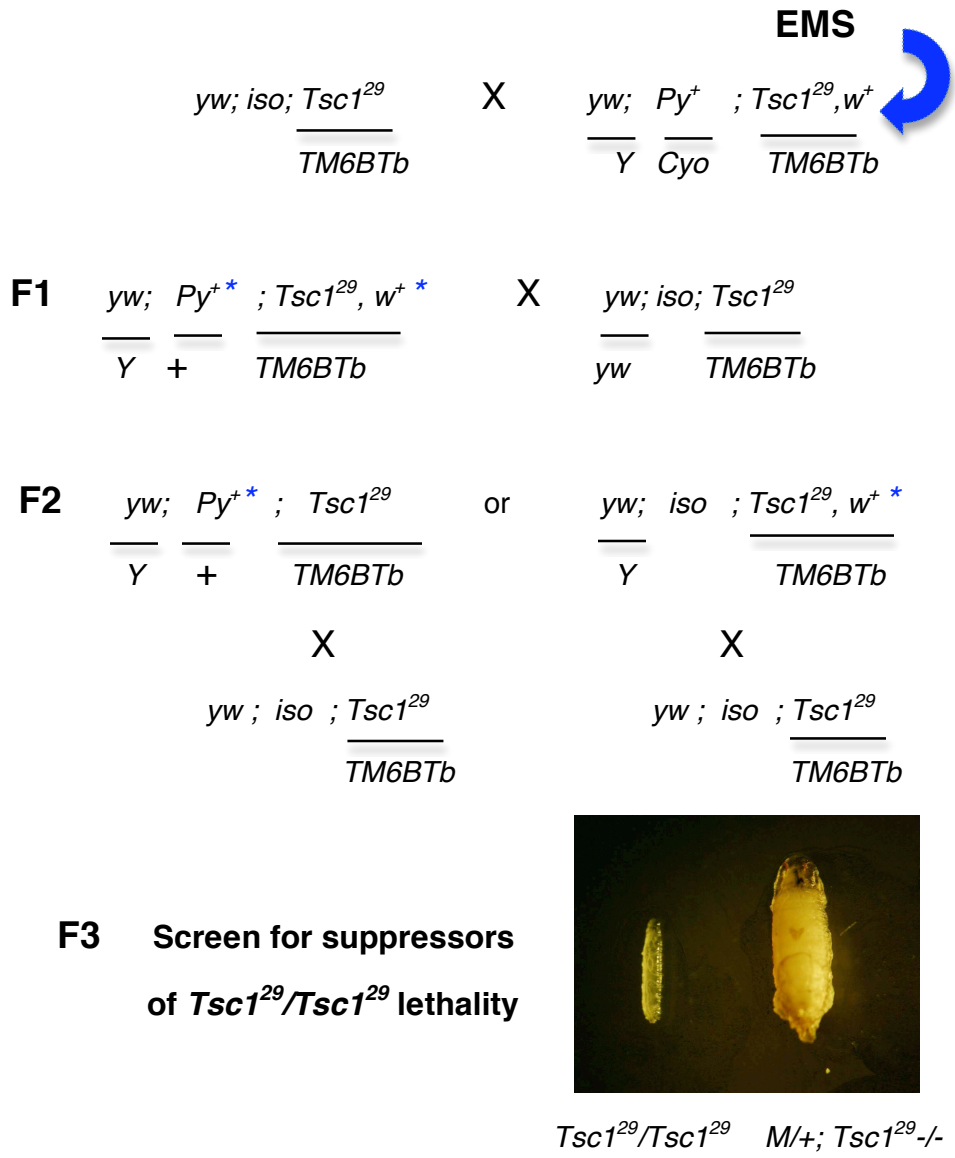


Figure 3C: Screening strategy for the desired EMS mutagenesis screen Parental males with marked second and third chromosome were mutagenized. All F1 males that inherited marked, mutagenized second and third chromosome were individually crossed to the female parental line. At the F2 level the two chromosomes were segregated and their F3 progeny was analyzed for at least partial suppression of early *Tsc1*^{29} lethality to the pupal stage. An example of a representative suppression effect is shown in the picture.

IV. Results of screening

Screening of 16,450 flies at the F2 level lead to the identification of 12 novel suppressors that partially rescued *Tsc1*^{29} early larval lethality in a dominant fashion (Table 3D). In addition, 11 alleles of TOR and 2 alleles of Rheb were obtained, providing a validation of the screening strategy and rationale. The large number of TOR alleles likely reflects the large size of TOR kinase, which spans about 280 kDa in size (Zhang Y et al., 2005). Eight of the twelve novel suppressors were embryonic or early larval lethal. Complementation tests were carried out between the second chromosome suppressors to identify multiple alleles of a complementation group while third chromosome suppressors could not be analyzed by complementation tests since they all carried the *Tsc1*^{29} null mutation in the background. However, each suppressor mapped to an individual complementation group. As mentioned earlier, one disadvantage of EMS screens is that they can generate multiple mutations on the same chromosome. Due to this complication, it was possible that the lethality of each mutant chromosome was due to presence of secondary lethal mutations rather than to the suppressor mutation itself, which was viable. To clean up the second chromosome of any secondary lethal mutations, I backcrossed the suppressors to a stock with an isogenised second chromosome and the third chromosome carrying the *Tsc1*^{29} null mutation, and followed the suppressor mutation by its suppression of *Tsc1* larval lethality. However in each case the suppressor remained linked to a

recessive lethal phenotype. These results suggest that each suppressor represents a mutation in an essential gene.

14 suppressors identified from screening 16,450 flies	
SECOND CHROMOSOME	THIRD CHROMOSOME
10 TOR alleles	2 Rheb alleles
M2*	M8*
M3*	M12*
M4*	M22*
M9	M23b+
M14	M24*
M16*	
M23a+	

Table 3D: Results of EMS mutagenesis screen with the identified novel suppressors of Tsc129 lethality.

* homozygous lethal phenotype

+ suppression seen only as an additive phenotype

We also analyzed genetic interactions between some of the suppressors and FRT-mediated loss of function clones of *Tsc1*^{29} in the eye. Loss of *Tsc1*^{29} function in the eye results in TOR hyperactivity, which translates to increased growth and size of the clonal population. The FRT-mediated clones in the eye were induced by *ey-flp.* Since the third chromosome was marked with a w^+ cell lethal marker, any surviving wild type eye tissue was marked red. The third chromosome suppressors M12 and M22 gave a significant and complete reduction of overgrowth observed in clones that lost *Tsc1*^{29} function respectively [Fig 3E]. Both M2 and M4, the two tested second chromosome suppressors, reduced the overgrowth seen in eye cell clones that had lost *Tsc1*^{29} function [Fig 3F].

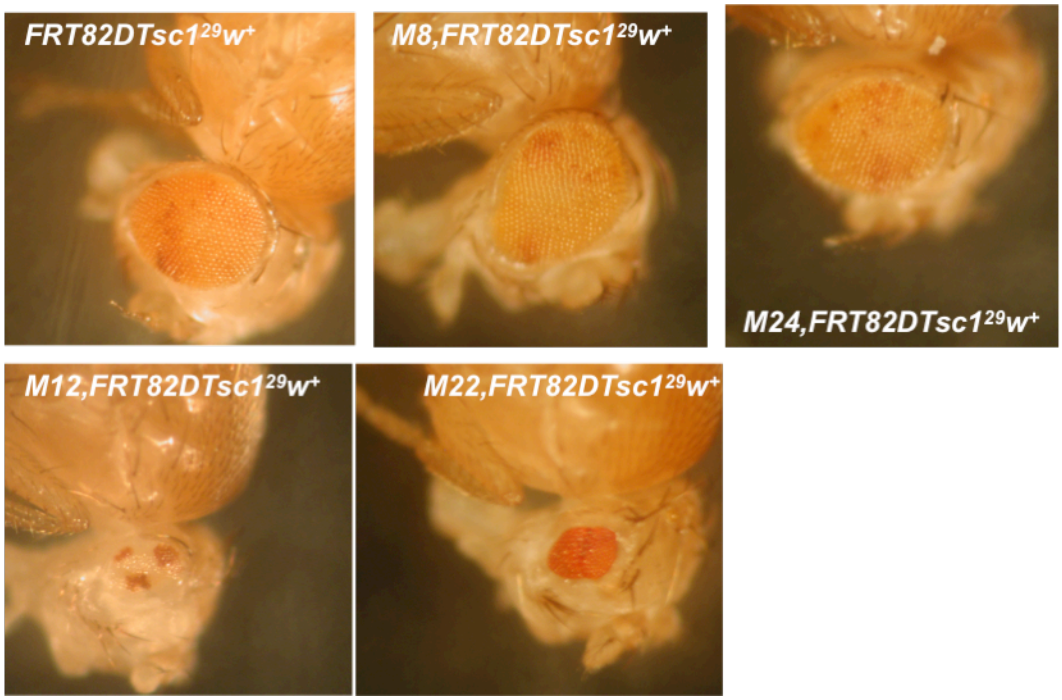


Figure 3E: Genetic interaction of third chromosome suppressors with *Tsc1^{29}* in the eye.

FRT-mediated *Tsc1^{29}* loss-of-function mitotic clones were induced in the eye using *ey-flp*. Since the third chromosome also carried a w^{+} cell lethal marker in addition to *Tsc1^{29}*, wild type eye tissue is marked in red. M8 and M24 did not show any significant difference in overgrowth seen in the *Tsc1^{29}* loss-of-function mitotic clones while M12 and M22 gave a significant reduction of *Tsc1^{29}* clonal overgrowth, indicating stronger genetic interactions.

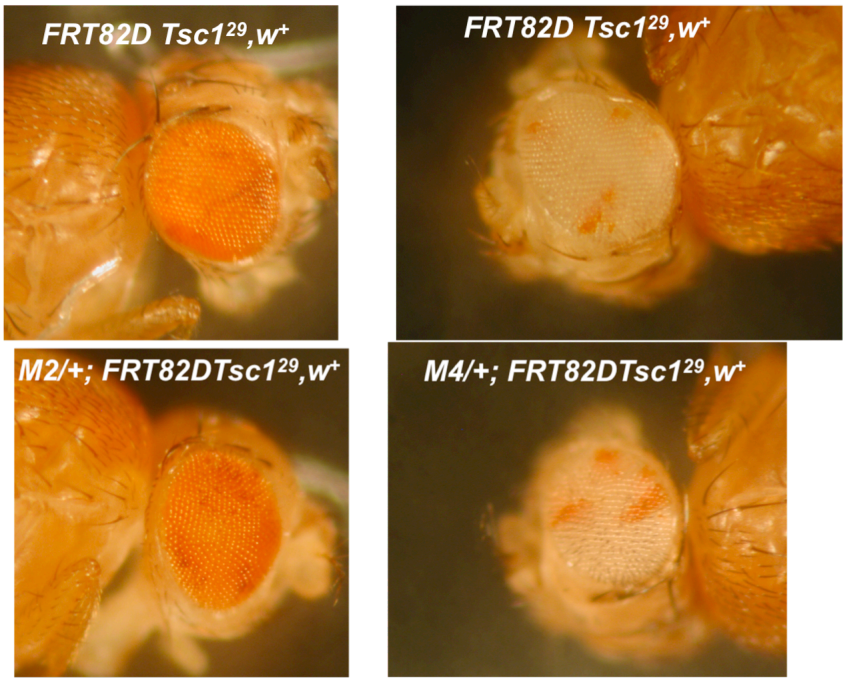


Figure 3F: Genetic interactions of second chromosome suppressors with Tsc1^{29} in the eye.

FRT-mediated *Tsc1^{29}* loss-of-function mitotic clones were induced in the eye using *ey-flp*. Since the third chromosome also carried a w^{+} cell lethal marker in addition to *Tsc1^{29}*, wild type eye tissue is marked in red. Both M2 and M4 show any slight reduction in the overgrowth seen in the *Tsc1^{29}* loss-of-function mitotic clones.

V. Mapping strategies and results

The historical use of *Drosophila* as a model for genetic and developmental biology analyses and the availability of the *Drosophila* genomic map provide a number of resources for mapping and mutagenesis. Large and small chromosomal deletions spanning the entire *Drosophila* genome have been created for use of mapping and characterizing mutations (Parks AL et al., 2004). These deletions, termed deficiencies, have been cytologically and/or molecularly mapped to defined endpoints using transposable P-element insertions. One can use the large cytologically defined deficiencies to crudely map test mutations to a rough genetic locus followed by the use of either the small molecularly defined overlapping deficiencies or the molecularly defined P-element insertional mutants for narrowing down this locus to an even smaller region. We used two sets of cytologically defined deficiencies from Bloomington Stock Center that provide about 57-79% coverage of the second chromosome and 77-79% coverage of the third chromosome (Wilson RJ et al., 2008). These Deficiency Kits were tested for complementation for three of the strongest second chromosome suppressors (M2, M3 and M4) and four of the third chromosome suppressors (M8, M12, M22 and M24). As described above, it was assumed that the suppressor mutations lead to lethality and it was not an effect of any secondary lethal mutations, though that remained a possibility. If we could map these to one deficiency, using the

same deficiency to test our screen rationale and its ability to partially rescue *Tsc1*^{29} early larval lethality would confirm that the suppressor indeed mapped to that particular locus. Since the third chromosome deficiencies did not have *Tsc1*^{29} in the background, this also provided a way to test if they mapped to one deficiency, suggesting that they were multiple alleles of one complementation group.

The third chromosome suppressors M8, M12, M22 and M24 were crossed to the third chromosome deficiency kit, and each was found to fail to complement the lethality of two distinct deficiency regions in addition to the Tsc1 locus [Fig 3G2]. These genetic deletions did not overlap and were physically distant from each other, suggesting that these suppressor chromosomes each carry two distinct lethal mutations. Alternatively, as the deficiency stocks vary greatly in their genetic background, it is also possible that additional lethal mutations reside outside of the defined deficiency interval, or that these chromosomes may interact with the *Tsc1*^{29} mutation or with EMS-generated mutations.

Complementation of the second chromosome suppressors with the second chromosome Deficiency Kit yielded three well-defined genetic regions where M2, M3 and M4 mapped [Fig 3G1]. Using 10, 14 and 23 smaller deficiencies that spanned and overlapped with the potential region for M2, M3 and M4 respectively, we tried to narrow down the cytological region while searching for interesting candidate genes within the identified genetic intervals.

However, some of the deficiencies from the stock center are not precisely mapped to the molecular level and hence their endpoints are not well defined. As a result, these overlapping regions may not provide precise information for finer mapping. In our screen, I could not find a common region within the deficiencies that failed to complement M4 and that was outside the cytological region of the deficiencies that complemented M4. With respect to M2 and M3 we could narrow the region down to a common overlapping cytological region between 58D1-59AB and 49F1-F10 respectively.

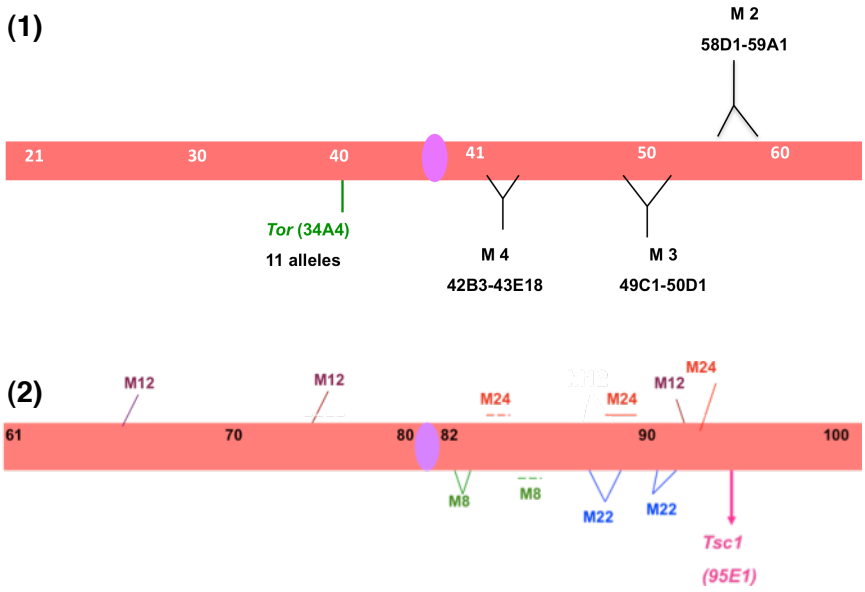


Figure 3G: Results of suppressor mapping using the Bloomington Deficiency Kit.

Using a second chromosome deficiency kit from Bloomington, rough mapping of three second chromosome suppressors by complementation tests gave distinct chromosomal loci as shown in (1), map not to scale. Cytological locations that M2, M3 and M4 mapped to are 58D1-59A1, 49C1-50D1 and 42B3-43E18 respectively. Complementation tests of the third chromosome suppressors using the third chromosome deficiency kit mapped them to more than one, distinct, non-overlapping loci. As shown in (2), M8 mapped to 82A1-82D3, 86C7-86E11 and 85A5-85B2, M12 mapped to 66B8-66C10, 75A6-75C2 and 92F7-93B6, M22 mapped to 88E7-89A1 and 90F1-91F5, and M24 mapped to 83E1-84B1, 84D4-85B6, 88E7-89A1 and 93B6-93D4.All third chromosome suppressors also failed to complement a Deficiency covering *Tsc1*

M2 mapped to a common region between two deficiencies, Df(2R)X58-12 and Df(2R)59AB. Genetic interactions of M2, which was the strongest dominant suppressor on the second chromosome were also tested with TOR overexpression phenotype. *TOR* overexpression in the eye using *ey-GAL4* results in a dominant negative effect and a reduced rough eye phenotype (Hennig K et al., 2002). The two adjacent deficiencies that failed to complement M2 using Deficiency mapping with the second chromosome kit, namely #282 [Df(2R)X58-12] and #590 [Df(2R)59AB] produced a rescue of the small and rough eye phenotype seen on eyGAL4>UAS-TOR expression [Fig 3H].

We also searched for obvious candidates within all the deficiencies that any of the suppressors roughly mapped to. One candidate that stood out was *partner of paired* (*ppa*) which lay in the overlapping region between two deficiencies that fully or partially failed to complement M2, namely 58D1-59AB. However, *ppa* mutation by itself did not suppress $Tsc1^{29}$ early larval lethality and also complemented M2. It also did not exhibit any change in TOR activity towards starvation-induced autophagy and cell size as measured by LysoTracker assay.

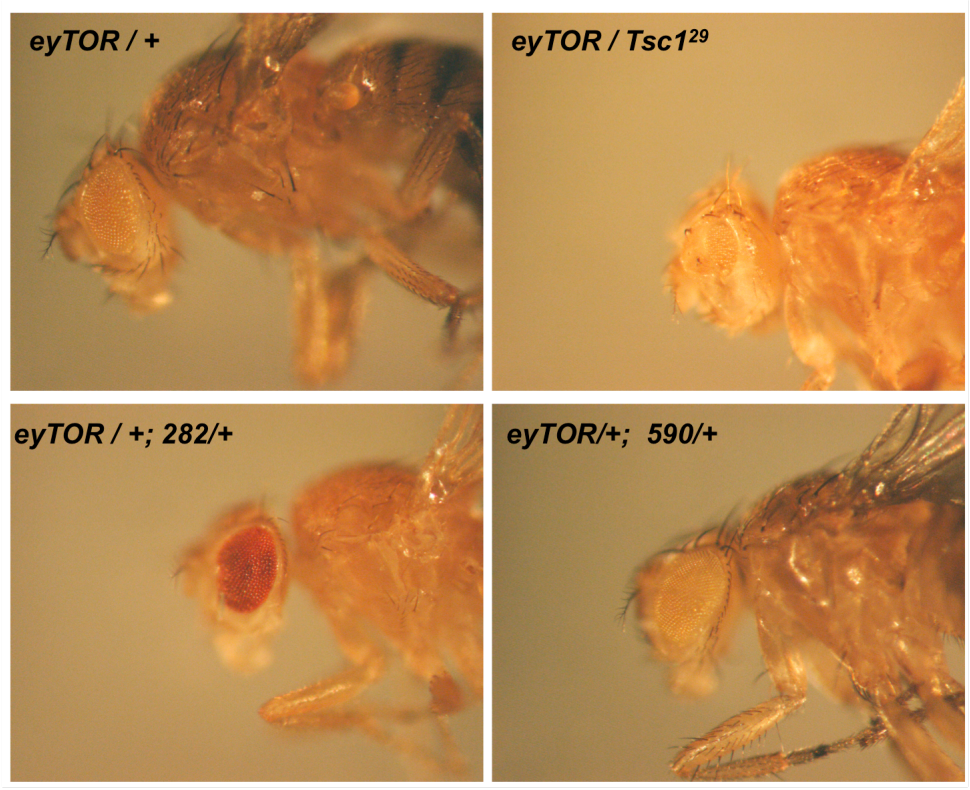


Figure 3H: Genetic interaction between potential M2 cytological region and TOR.

TOR overexpression in the eye using *eyGAL4* results in an small sized rough eye (eyTOR/+). Reducing dosage of $Tsc1^{29}$ in an eyTOR background leads to hyperactive TOR signaling resulting in a smaller and rougher eye phenotype. Amongst the deficiencies 282 and 590, that failed to complement M2 either fully or partially respectively, 590 can significantly rescue the eye size but not the roughness while 282 can weakly rescue eye size and not rough phenotype of is. The above pictures are a representation of male eye phenotype of the noted genotypes ($n \geq 5$).

M3 mapped to an overlapping region between Df(2R)CX1, Df(2R)vg-b and Df(2R)Exel8057. Within the common cytological region 49F1-49F10 that M3 mapped to we tested available stocks of genes for complementation with M3 such as *sieve*, *suppressor of zeste(3)*, and 6 lethal genes of unknown function, which exhibited complementation. Even though we could not narrow down the region for M4 we tested the following candidate genes mapping to the deficiencies that failed to complement M4 – *sine oculis, KBras, crib, CG30502*, *maternal effect cellularisation defect J, satin and humulis* respectively. Amongst these, only *sine ocoulis* (*si*) failed to complement M4. However, two different stocks of *si* failed to suppress $Tsc1^{29}$ early larval lethality. This suggests that M4 is distinct from si and the observed failure of complementation could arise due to shared lethal mutations on the second chromosome or genetic interactions between M4 and *si*.

Since our Deficiency mapping strategy was unsuccessful in accurately narrowing down the suppressors to a smaller cytological locus and in finding suppressors that were multiallelic, we decided to change mapping strategies.

Another way of mapping mutations is P-mediated recombination mapping by using molecularly defined P-element insertions that span the desired chromosome (Zhai R et al., 2003). This allows for mapping to the precise molecular interval and provides a position of the test mutation relative to the P-element insertion site. However, this is a very stepwise and time-consuming

process that needs visible markers and high numbers to determine the exact recombination frequency (about 100 or more). In our case, the visible marker was the partial rescue of *Tsc1*^{29} early larval lethality to the pupal stage. This meant that all the P-insertions to be used had to be redesigned in a *Tsc1*^{29} background, a step that took two generations. Taking this into account, I decided to focus on using the above strategy for P-mediated recombination mapping of M2, the strongest rescuer of *Tsc1*^{29} larval lethality to roughly map it to a smaller molecularly defined region and then repeat the same strategy for finer mapping. M2 had mapped to a relatively distinct region with deficiency mapping and had shown genetic interactions with eye specific *Tsc1*^{29} loss of function and *TOR* overexpression, which justified putting effort into its mapping.

In an unbiased manner, 12 equidistant P-element insertional mutants (about 10 genetic map units apart) marked with w^+ element were selected on the second chromosome. Care was taken to ensure that these insertions were viable and had a similar genetic background. They were appropriately crossed so as to carry *Tsc1*^{29} null mutant on their third chromosome in order to use the ability of M2 to suppress *Tsc1*^{29} larval lethality to the pupal stage as a marker. Recombination was allowed to occur between each P-element insertion and M2. Each male progeny was checked for recombination events as determined by segregation of rescue of *Tsc1*^{29} larval lethality and inheritance or loss of w^+ element as the P-element marker. If the P-element and M2 failed to recombine, it

suggested that they were physically closer. If they were further apart on the chromosome, they were expected to show a higher rate of recombination [Fig 3I]. The results suggested that there was very low recombination (0-0.4%) between P-elements at cytological positions 31B1 and 34E6 hinting this might be the genetic region M2 maps to on the left arm of the second chromosome. This was not the same cytological region indicated by Deficiency mapping namely 58D1-59AB. It would, however, be expected since the P-element mapping approach mapped the suppressor phenotype whereas Deficiency mapping mapped the lethality, which might have arisen due to EMS-generated secondary lethal mutations.

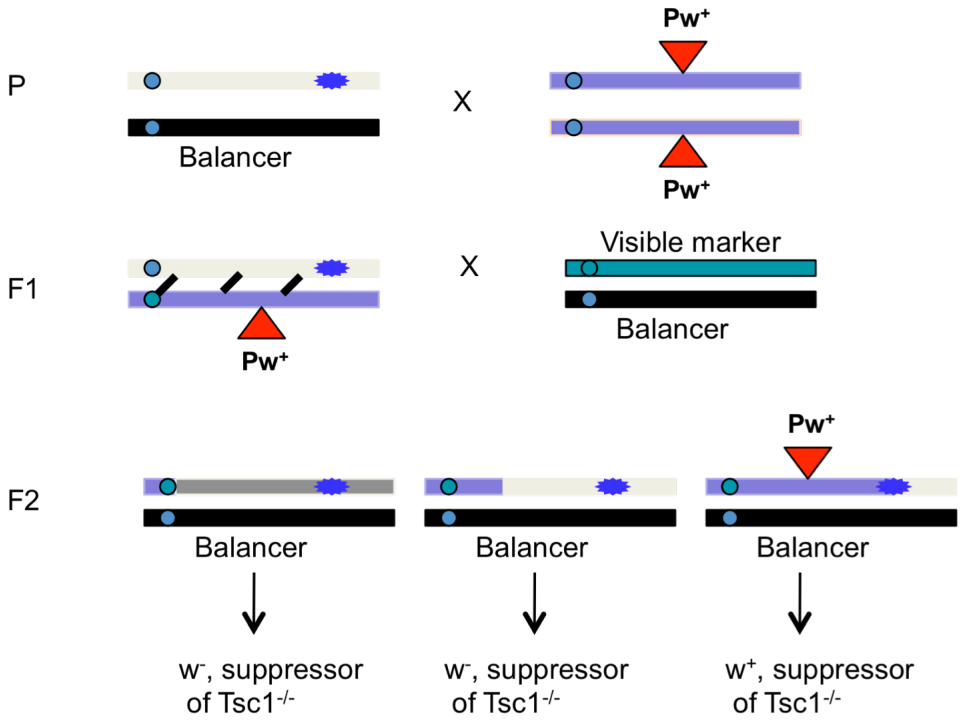


Figure 3I: Strategy for P-element mediated recombination mapping employed for M2 mapping.

Marked P-elements of known insertion points that were equidistant from each other were put in conjugation with a *Tsc1*^{29} null allele carrying third chromosome and used as parents. These were crossed to M2/balancer; *Tsc129*/TM6BTb to obtain the F1 females that would permit recombination between M2 and P-element. All potential recombined F2 males were collected and checked for recombination based on the ability to suppress Tsc1^{29} early larval lethality and the presence of w^+ marker belonging to the P-insert. A higher rate for recombination would be expected for P-inserts that were farther away from M2, whereas lower or no recombination would be observed when M2 and P-insert were closer on the chromosome.

VI. Limitations and open questions

Our forward genetic approach to identify novel regulators of TOR pathway, though validated by obtaining hits in known regulators such as TOR and Rheb, was not completely successful. We tried to employ an unbiased approach and hence preferred to use EMS as a mutagen. The main caveat for this study was that we were unable to confirm the lethality observed in the suppressors to the presence of the suppressor itself and not to the generation of secondary lethal mutations by EMS, inspite of the standard dosage used. If we had obtained more than one allele of a single complementation group, it would have been possible to both clean up the chromosome by putting these alleles in trans and continue finer mapping.

The suppressors by themselves did not exhibit any obvious phenotype except embryonic and early larval lethality. In order to see the phenotype of the presence of any of the suppressors, the *Tsc1*^{29} background was required, which complicated analysis for the third chromosome suppressors. This also curtailed the numbers that could be generated for P-mediated recombination mapping since it required a two-three generation cross to obtain the correct genetic background.

We did find strong genetic interactions between some of the suppressors and *TOR* overexpression as well as *Tsc1*^{29} clonal loss of function [Fig 3E, F and H]. However, Deficiency mapping of these suppressors yielded complicated

results [Fig. 3G]. I tried to focus on the strongest second chromosome suppressor M2, which roughly mapped to 2R region 58D-59AB, using the second chromosome deficiency kit. However, using a P-element mediated recombination approach to identify molecularly defined region, M2 roughly mapped to a region on 2L between 31B1 and 34E6. Since the P-mapping strategy follows the suppressor phenotype rather than lethality, as accomplished by Deficiency mapping, it is reasonable to assume that the former has more potential and holds true. In conjugation with the observation that one of the deficiencies that failed to complement M2 partially, #282 [Df(2R)X58-12], could not act as a suppressor of $Tsc1^{29}$, it suggests that the deficiency carries a secondary lethal, possibly generated by EMS resulting in the observed M2 lethality. Since #282 and #590 [Df(2R)59AB] overlap, even though #590 can suppress $Tsc1^{29}$ lethality, it may do so due to a shared lethal mutation in the background, mapping M2 to this deficiency. The P-element mapping strategy is thus more promising for mapping these suppressors and not any secondary lethal mutations. Due to time limitations, further refined molecular mapping is not possible with M2 or with the other second chromosome suppressors at this point.

The 12 novel suppressors that were identified in this study act as promising bonafide regulators of TOR activity. They can dominantly rescue *$Tsc1^{29}$* early larval lethality and reduce the resulting TOR hyperactivity to a similar extent as the known positive players in the pathway. It is possible and worthwhile

to map them molecularly using P-element recombination mapping though it will be a time consuming and labor-intensive project. Recently a genome sequencing method for the entire organismal genome has been developed in *C. elegans* to enable parallel sequencing and thereby genomic alignment and comparison between the genomic sequences of two strains of worms in a short time using a high through out Illumina Genome Sequencer (Hillier LW et al., 2008). This technique has tremendous potential to detect single nucleotide changes after mutagenesis and enable easier characterization of identified mutants. In future, this technique can be adapted for whole genome sequencing in *Drosophila*. This could aid the identification of the suppressors generated in the current screen in a shorter time frame with a higher accuracy.

CHAPTER 4: dRag GTPases ACT AS NOVEL POSITIVE REGULATORS OF TORC1

Parts of this chapter have been already published in the following publication:

Kim, E., P. Goraksha-Hicks, L. Li, T.P. Neufeld, and K.L. Guan. 2008. Regulation of TORC1 by Rag GTPases in nutrient response. *Nat.Cell Biol.* 10:935-945.

I. Introduction

Small GTPases are monomeric proteins, about 20-25kDa in size, that act as molecular switches to control a wide variety of functions such as cell growth, division and differentiation, cell movement and migration, vesicular transport, and nuclear import and export. RagA and RagC are the only two known members of a novel sixth family of small GTPases (Hirose E et al., 1998, Schürmann A et al., 1995). The Rag family is unusual among other small GTPases as it lacks the characteristic lipid modification domain seen within all other small GTPases. A second unique feature is that RagA and C both undergo homodimerization and subsequent heterotetramerization, presumably through their unusually long C-terminal domains (Schürmann A et al., 1995). This stable heterotetrameric complex formation occurs in a nucleotide independent manner and is crucial for Rag function. In yeast, GTR1 (yeast RagA) has been shown to exist in a GTP-bound state while GTR2 (yeast RagC) is present in a GDP-bound state (Gao M and Kaiser C, 2006). One can therefore speculate that RagA/B acts as the catalytically active component while RagC/D contributes to the stability of the complex and/or functions as a binding partner for complex formation with regulators and effectors, to achieve downstream signaling.

Drosophila RagA (dRagA) and RagC (dRagC) is most homologous to mammalian RagA/B and RagC/D respectively and less so to the yeast GTR1 and

GTR2 respectively. GTR1 was first identified in *Saccharomyces cerevisiae* as important for inorganic phosphate uptake via functional association with Pho84, the inorganic phosphate transporter (Bun-ya M et al., 1992). Mammalian RagA/B were identified as homologs of the yeast GTR1 (Hirose E et al., 1998). Around the same time studies implicated that GTR1 negatively regulated the Ran-GTPase cycle possibly through GTR2 (Nakashima N et al., 1999). Later the exact mechanism was discovered that Gtr1p & Gtr2p competitively bind away the Ran-GAP binding protein, Yrb2p, thus allowing the freed Ran-GAP to negatively regulate the Ran cycle (Wang Y et al., 2005).

Mammalian Rag A as a part of the Rag GTPase complex was also found to interact with a novel nucleolar protein Nop132 using yeast two hybrid screen, implicating role for Rag GTPases in rRNA transcription and processing (Sekiguchi T et al., 2004). The first major link between Rag GTPases and TOR activity came from investigations in yeast that identified GTR-GTPases to be important for TOR-regulated functions. GTR-GTPases, along with GSE complex members, regulate general amino acid permease 1 (Gap1) sorting to the plasma membrane on nutrient limitation (Gao M and Kaiser CA, 2006). In a parallel study both GTR GTPases, as a part of EGO complex, and TOR were shown to positively regulate microautophagy (Doubouloz F et al., 2005). In this same study GTR-GTPases were also found to be important in regulating exit from rapamycin-

induced cell-cycle growth arrest. These studies implicate an overlap and therefore a connection between Rag and TOR activity.

Due to the importance of the small GTPase Rheb as a direct upstream regulator of TOR kinase, a RNAi screen was performed in *Drosophila* S2 cells to identify other small GTPases that were necessary for TOR activity as measured by S6K and 4EBP phosphorylation (Kim E et al., 2008). Knockdown of dRagA and dRagC was found to decrease TOR activity though not to the same level as Rheb knockdown.

All the above studies implicated that Rag-GTPase activity was conserved among eukaryotes for normal cellular growth via regulation of anabolic processes such as nucleocytoplasmic transport, ribosome biogenesis, regulation of amino acid transporter sorting, microautophagy, exit from G_0 state and translation. Using a reverse genetics approach, we identified dRag GTPases as potential positive players in the TOR pathway. A genetic analysis of whether dRags are important for TOR activity and regulation forms the basis of the current study.

II. *dRagC* acts as dominant suppressor of *Tsc1*^{29} lethality

Tsc1^{29} homozygous null animals die due to TOR hyperactivity. We have previously used the dosage sensitive genetic interactions in the TOR pathway to design a forward genetic screen and identify positive players, based on their ability to act as partial suppressors of Tsc1^{29} early larval lethality. We utilized this assay as an *in vivo* test of the potential role of dRag genes in TOR signaling. We

identified a lethal P insertion (EY11726) in the first exon of *dRagC*, and validated this mutation by showing that excision of the element resulted in complete viability. The P-insertional mutant of *dRagC*, hence forth referred to as *dRagC*, acts as dominant suppressor of $Tsc1^{29}$ lethality and partially rescued the $Tsc1^{29}$ early larval lethality to the pupal stage in 7.2% animals (n=474), [Fig. 4A-1, 2]. This highly supports involvement of Rag GTPases as novel positive regulators in the TOR pathway and provides evidence that Rag GTPases act downstream to or in parallel with TSC complex to activate TOR signal.

The P-insertional *dRagC* mutants undergo growth arrest as third in-star larvae, which is similar to that seen in TOR homozygous null larvae. This highlights a requirement of endogenous *dRagC*, and thereby Rag GTPase complex, in cell growth regulation and development.

(1)

Tsc1^{29}/Tsc1^{29} ***dRagC/+; Tsc1^{29}/Tsc1^{29}***

(2)

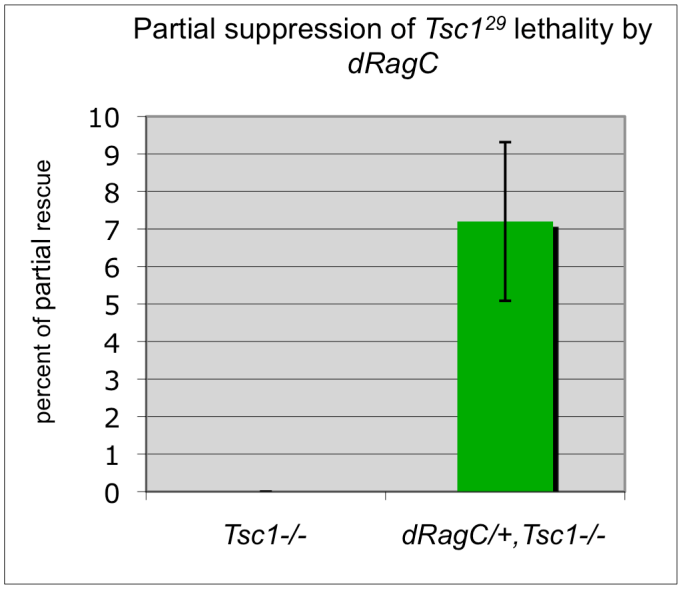


Figure 4A: *dRagC* acts as a positive regulator of TOR activity.

dRagC acts as a dominant suppressor of *Tsc1^{29}* larval lethality. *Tsc1^{29}* homozygous null mutants die in early larval stages as seen in (1). A mutation in *dRagC* in this background can partially rescue this lethality to the pupal stage in 7.2% animals (2). [n=474]

III. dRag-mediated regulation of wing growth & size

The TOR pathway has been well established as a positive regulator of growth and cell size in *Drosophila*. A characteristic feature of the TOR pathway and its members is to positively regulate individual cell size, which is not affected by general growth promoting genes such as the ribosomal *Minute* genes. This led me to examine the *in vivo* contribution of Rag GTPases for organ and cell size control. Transgenic *Drosophila* lines expressing wild type, constitutively active and dominant negative *dRagA* (*dRagA WT, dRagA Q61L* & *dRagA T16N*) and *dRagC* (*dRagC WT*, *dRagC Q99L* & *dRagC S45N*) under UAS control were constructed and crossed with the appropriate GAL4 lines to enable target gene expression. The effect of *dRagA* and *dRagC* was first examined in *Drosophila* wing by expressing wild type, constitutively active and dominant negative *dRag* transgenes in the dorsal wing compartment using dorsal specific driver *ap-GAL4*.

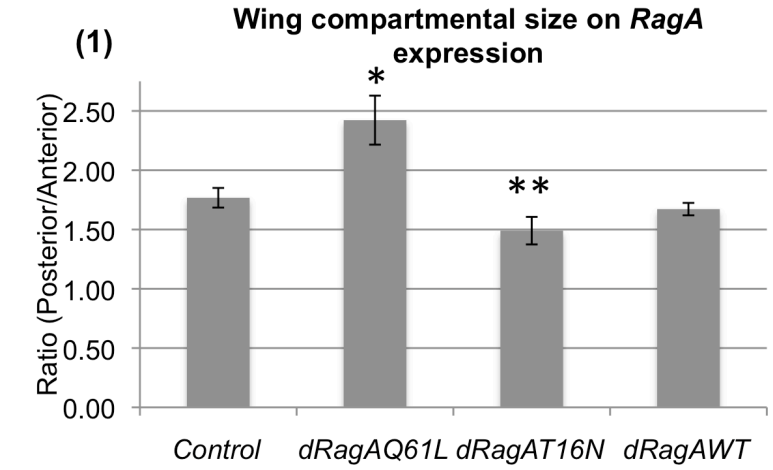
(1) *apGAL4>dRagAQ61L* **(2) *apGAL4>dRagT16N***

Figure 4B: *dRagA* promotes wing growth.

Expression of *dRagAQ61L* (1) or *dRagAT16N* (2) in the dorsal wing region exhibits convex wing curvature (increased growth) and concave wing curvature (decreased growth) respectively, suggesting a role for dRagA as growth promoter.

While *dRagC* expression did not show any effect (data not shown), *dRagA* expression positively regulated dorsal compartment size. Overexpression of constitutively active *dRagA* lead to a convex curvature of the wing resulting from overgrowth of the dorsal compartment whereas overexpression of dominant negative *dRagA* resulted in an upward wing curvature arising from decreased size of dorsal wing compartment. [Fig.4B].

In order to quantify the actual effect on wing compartmental size, *dRag*-transgenes were expressed in the posterior wing compartment using *en-GAL4*. Constitutive expression of dRagA resulted in an increase of the posterior compartment size relative to the anterior compartment. Consistently, reducing dRagA activity by expressing dominant negative *dRagA* decreased posterior compartment size relative to the anterior compartment [Fig 4C1]. The effect on organ size was also consistent with effect of dRag-expression in individual wing cells. Higher activity of dRagA promoted an increase in individual wing cell size while reduced dRagA activity decreased individual wing cell size [Fig 4C2].



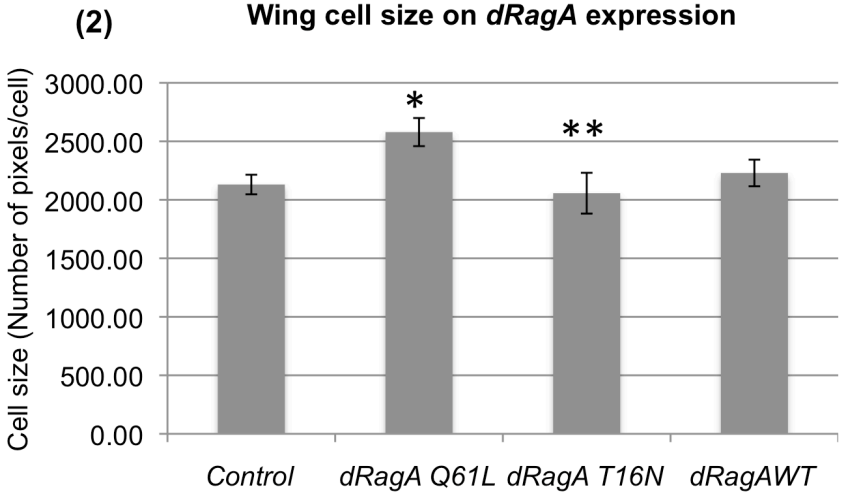


Figure 4C: *dRagA* positively regulates wing growth and cell size. (1) Wild type or mutant dRagA transgenes were expressed in posterior compartments with the en-GAL4 driver. The ratios of representative posterior to anterior compartment areas are shown in the bar graph. Posterior compartmental size is significantly increased on *dRagAQ61L* expression and decreased on *dRagT16N* expression. Data are mean ± s.d.; P-values: $*7.32 \times 10^{-4}$ [n=7], $** 6.18 \times 10^{-4}$ [n=12]; Student's 2-tailed t-test. (2) Average cell size in a representative posterior compartment expressing different *dRag* transgenes under control of *en-GAL4* is shown. Individual wing cell size in *dRagAQ61L* expressing region is increased relative to wild type while *dRagT16N* expressing cells are smaller in size. Data are mean ± s.d.; P-values: $*1.15 \times 10^{-3}$ [n=7], **0.025 [n=12]; Student's 2-tailed t-test. [n = number of wings analysed].

dRagC expression also positively contributed to organ and individual size. Consistent with the observation that RagC is active in a non-GTP bound form (Gao M & Kaiser C, 2005, Kim E et al., 2008), it was observed that nucleotide free dRagC increased both posterior wing compartmental size relative to the anterior compartment (Fig.4D1) as well as individual cell size significantly (Fig.4D2). GTP-bound dRagC expression decreased both posterior wing compartmental size relative to the anterior compartment (Fig.4D1) and individual wing cell size (Fig.4D2) within the posterior compartment slightly, though this decrease was not significant. This strengthens the concept that dRagC is the stabilizing member of the dRag GTPase complex while dRagA is the catalytically active member.

The above data indicate that Rag GTPases positively contribute not only to overall organ growth but also at the individual cell size level, reaffirming their novel status as TOR-pathway member. Highly active Rag GTPases promote growth while inactive RagGTPase inhibit growth.

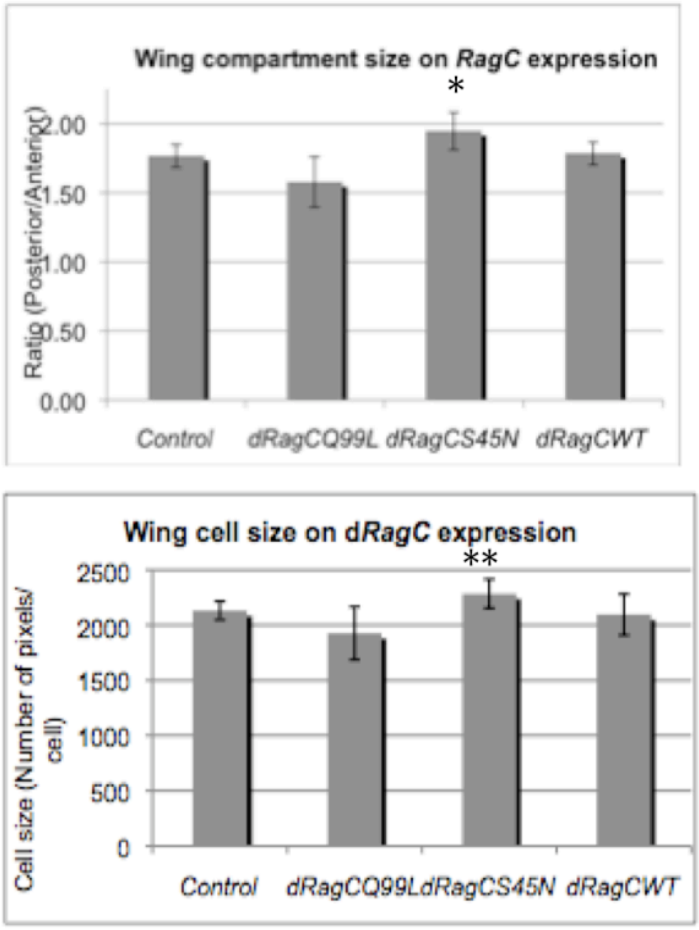


Figure 4D: dRagC promotes wing growth and cell size independent of its nucleotide status.

dRagC catalytic activity is not required for growth promoting effects in the wing. Expression of nucleotide free *dRagCS45N* using *enGAL4* significantly increases both posterior wing compartmental size increase (1) and individual cell size within the posterior compartment (2). Mean data ± s.d.; P values = * 8.57 x 10-5, **0.05; Student's 2-tailed t-test; n=13. Expression of the GTP-bound dRagCQ99L shows inhibitory effect on both posterior wing compartmental size (1) as well as individual cell size (2) relative to control, n=12. Expression of *dRagCWT* exhibits effects similar to control, n=11 (n= number of wings analyzed).

It is well known that TOR activity is downregulated on nutrient limitation. Therefore, I investigated how starvation, particularly for amino acids, would affect the compartmental growth and cell size effect seen on *dRag*-expression. Early third instar larvae were starved on 20% sucrose until pupation and wing growth effects were quantified post-eclosion.

The increased posterior compartmental and wing cell size growth seen on constitutive *dRagA* expression is decreased after larval starvation, though not significantly relative to control posterior wing compartment and individual cell size [Fig.4E1]. Similarly, a slight and insignificant decrease of dominant negative *dRagA* expressing wing cell size was observed post-starvation. However, posterior compartmental size remained the same [Fig 4E2]. No effect on wing compartmental growth and cell size effects on *dRagC* expression were observed post-starvation (data not shown).

The wing appendage is not involved in nutrient sensing or uptake, which may be the reason why starvation does not cause significant growth effects in this organ. This can also explain why no difference in eye size was observed on eye-specific expression of different *dRagA* constructs using GMR-GAL4 driver (data not shown). Therefore, it was important to study how dRags contributed to growth in a tissue that is sensitive to starvation and nutrient limitation.

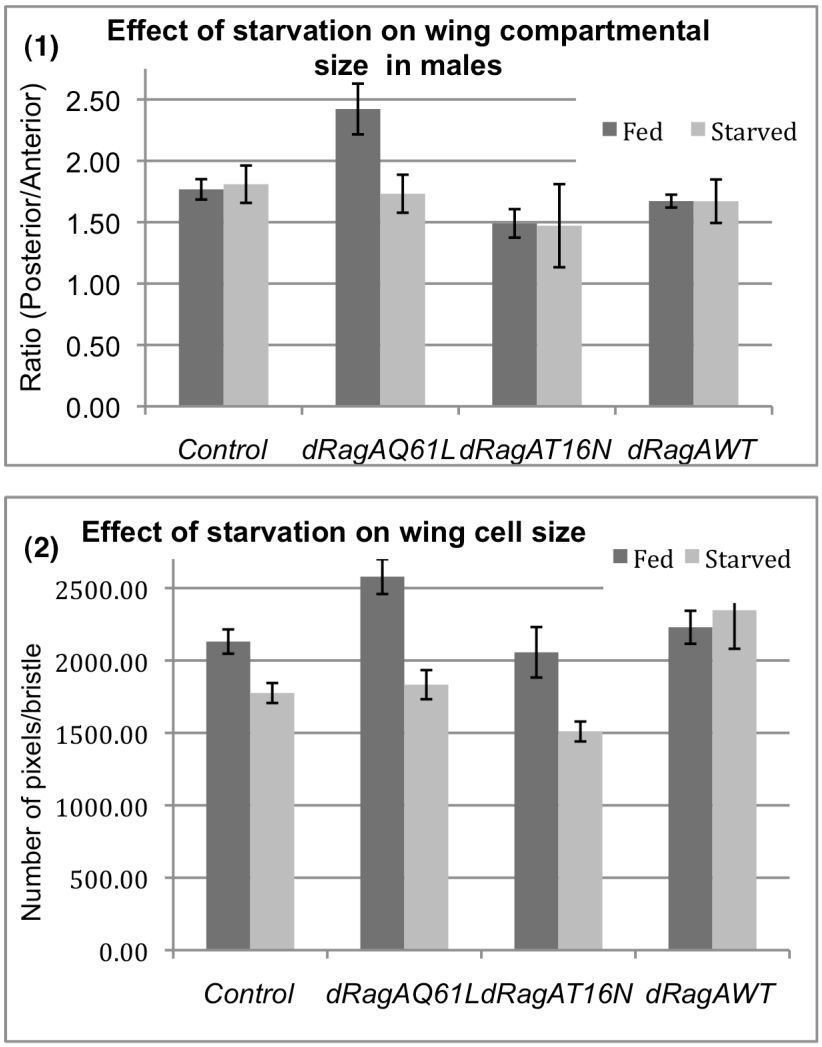


Fig 4E: dRagA activity is inhibited on amino acid starvation in the wing.

The increased compartmental (1) and individual cell size (2) seen on *dRagAQ61L* expression in the posterior wing compartment using enGAL4 undergoes a decrease after starvation for amino acids. This decrease is not significant relative to the decrease seen in the wild type wing compartmental and wing cell size (n=6). No significant change was seen with *dRagAT16N* and *dRagAWT* expression post starvation (n=16 and 4 respectively where n= number of wings analyzed.

IV. dRag-mediated regulation of fat body cell size

Drosophila fat body is an endocrine tissue that is involved in TOR-dependent nutrient sensing and in relaying nutritional response (Colombani J et al., 2003). Due to this important role, it is a tissue where TOR activity is well regulated, which makes it an ideal tissue to study TOR regulation with respect to nutrient availability and sensing. We looked at fat body cell size as readout of TOR activity under conditions of nutrient sufficiency and amino acid starvation for 48 hours on *dRag* expression in third instar larvae.

Under conditions of nutrient sufficiency, dRag GTPase inactivation by clonal overexpression of dominant negative *dRagA* significantly decreased fat body cell size as measured by Phalloidin assay. No effect on cell size was observed in clones with highly active dRagA (cells with *dRagA* wild type or *dRagA* constitutive active expression) under nutrient sufficiency [Fig. 4F1]

However, in an amino acid depleted background, cells expressing *dRagA* wild type were significantly increased in size and there was a dramatic (three-fold) increase in size of fat body cells with constitutively active *dRagA* expression [Fig. 4F1,2]. Cell size of dominant negative *dRagA* overexpressing clones did not decrease on amino acid starvation but only in nutrient sufficiency [Fig. 4F1]. Since dominant negative dRagA showed effect on cell size only under nutrient sufficiency [Fig. 4F1,2] and not limitation, these data strongly support that dRagA plays a role in nutrient, especially amino acid, sensing.

Overexpression of *dRagC* did not alter fat body cell size significantly [Fig4F1], which is similar to the results seen in mammalian cells where RagC weakly regulated mTORC1 (Kim E et al, 2008, Sancak Y et al, 2008). To test the requirement of *dRagC* for TOR regulation, fat body cell size was determined in FRT-induced loss of function clones of dRagC using the P-insertional *dRagC* mutant. Interestingly this *dRagC* mutation resulted in a modest but statistically significant decrease of cell size under well fed conditions, highlighting the endogenous role of *dRagC* in growth regulation [Fig 4F1,2].

Taken together, the above data reveal an endogenous and important role for *dRag* GTPases in growth regulation and nutrient sensing.

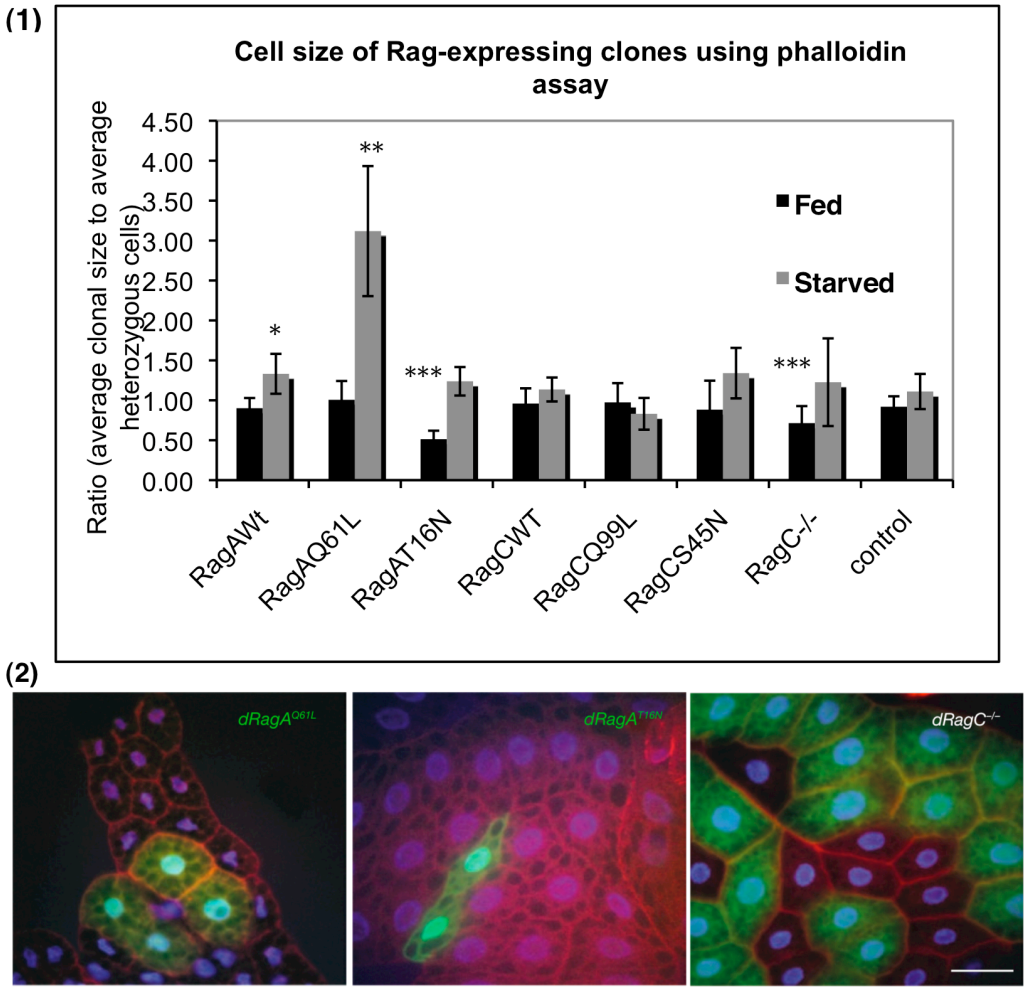


Figure 4F: dRags regulate fat body cell size in *Drosophila* larvae.

(1) The bar graph shows cell size of clonally induced *dRagA* expressing cells or *dRagC* homozygous mutant cells relative to neighboring wild type control cells in larval fat body. Fat body cell area of well-fed or 48-hour starved larvae was determined using Phalloidin staining [(2) in red)]. Decreased dRag activity by *dRagT16N* expression and in *dRagC* loss of function clones results in smaller cell size relative to control cell size, under nutrient availability. Expression of *dRagQ61L* and *dRagAWT* results in significant increase in fat body cell size on nutrient limitation. Data are mean ± s.d.; P values = *2.04 x 10-3 (n=5), **2.94 x 10-6 (n=14), ***3.79 x 10-7 (n=14), ****1.36 x 10-5 (n=30), Student's 2-tailed t-test. (2) Representative images of Rag-mutant fat body cells where GFP marks *Rag* transgene expression and for *dRagC* homozygous mutant, loss of function clones are marked bv loss of GFP.

V. dRag GTPases play an important role in starvation response

Organisms need to be able to orchestrate a proper starvation response on nutrient limitation. This involves turning down growth and anabolic processes, and shifting metabolism from storage and generation of energy storing molecules, such as fats, to catabolism of these energy rich deposits. In *Drosophila* larvae, autophagy is robustly initiated in the fat body as a starvation response on a starvation period as low as 3 hours on 20% sucrose. This starvation-induced autophagy can be visualized by the staining of the expanded autolysosomal compartment by the acidic dye LysoTracker. Clonal overexpression of constitutively active d*RagA* completely blocked starvation-induced autophagy in *Drosophila* fat body [Fig. 4G(a)]. This indicates that active dRagA suppresses the nutrient starvation response, suggesting that high dRagA activity may generate false signals mimicking nutrient sufficiency to the cells, thereby suppressing autophagy.

No effect on starvation-induced autophagy was observed on expression of dominant-negative and wild type *dRagA* as well as wild type, constitutive active and dominant negative *dRagC*. This can be explained by the observation in mammalian cells where RagA is more dominant over RagC for S6K phosphorylation (Kim E et al., 2008).

While the LysoTracker assay analyzes the final step of autophagy, the dye's acidic affinity can provide non-autolysosomal staining. Hence, a more

definitive test is to look at another step like initiation of autophagy induction. During induction of autophagy, Atg8 (mammalian LC3) gets conjugated to phosphatidylethylamine and is incorporated into the assembling autophagosomal membrane (Kabeya Y et al., 2000, & Kabeya Y et al., 2004). The localization of GFP-tagged Atg8 can therefore be used as a direct measure of autophagic activity. To confirm the complete block of starvation-induced autophagy observed in clones with highly active RagA, I analyzed Atg8a-GFP localization in clones expressing different *dRagA* and *dRagC* constructs. As expected post-starvation, the number of Atg8A-GFP-positive punctae is significantly decreased in clonal cells with highly active dRag GTPase complex expression. Under well-fed condition where normally autophagy is inhibited, cells with inactive dRag GTPase complex display a significant increase in the number of Atg8a-GFP positive autophagosomes relative to control cells [Fig. 4G(b-d)].

Thus dRag GTPase complex plays an important role in nutrient sensing and in conduction of starvation response.

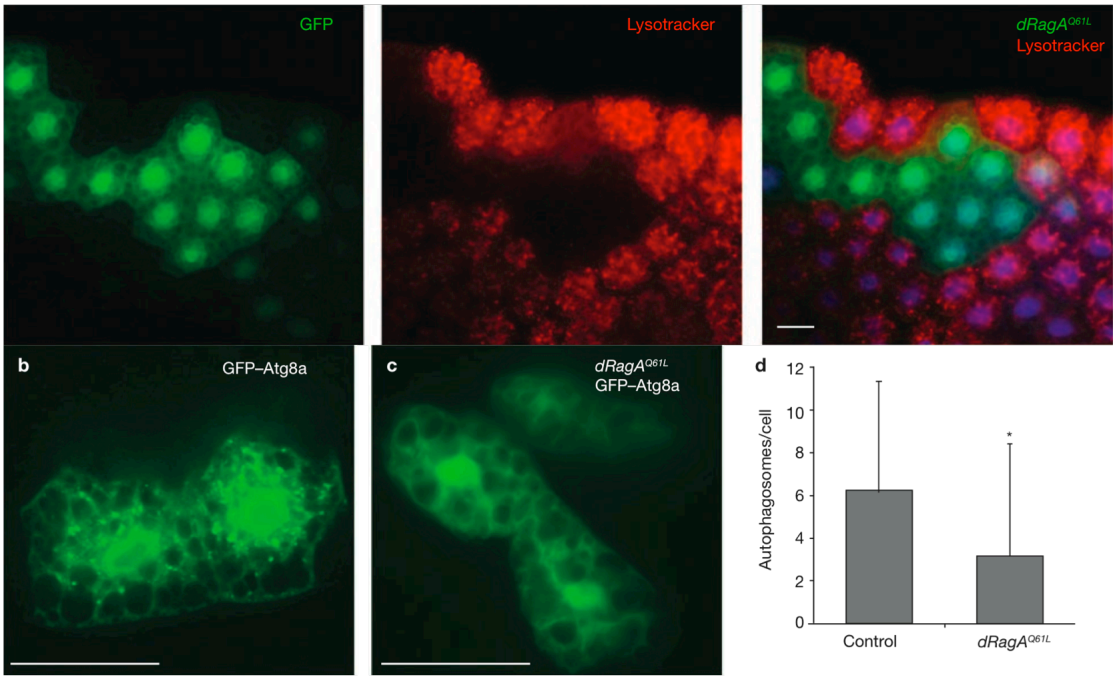


Figure 4G: dRagA inhibits starvation induced autophagy.

(a) *dRagAQ61L* expressing fat body clonal cells (marked by GFP expression) completely inhibited starvation induced autophagy (marked by autolysomes that incorporate the red dye LysoTracker). Autophagy induction is marked by the number of Atg8a-GFP positive punctae in control cells (b). Induction of autophagy is significantly decreased in *dRagAQ61L* expressing clones (c). (d) Average number of Atg8a-GFP positive punctae per cell in control and *dRagAQ61L* expressing fat body clonal cells is plotted.

VI. dRag GTPases regulate organismal metabolism

The data thus far point to the importance of dRag GTPases in nutrient sensing and growth at a cellular level. It was necessary to investigate how dRags regulate co-ordination of metabolism at the organismal level. A proper response to starvation is necessary for organisms to tide over unfavorable nutritional conditions, generally manifested by undergoing autophagy. Therefore, we looked at adult viability in response to complete starvation. As expected, adults with a high dRagA activity, either ubiquitous [Fig 4H2] or fat body specific [Fig 4H3], are more sensitive to death by starvation as compared to control adults, while adults with reduced dRagA activity are more resistant to death by starvation. Though a similar trend is seen in well-fed control adults for a similar time period [Fig. 4H1-3], the susceptibility to starvation seen on constitutive activation of *dRagA* in the fat body under complete starvation is significant.

This is suggestive of the importance of dRag GTPases in sensing and relaying nutritional response, and thereupon affecting organismal metabolism and viability.

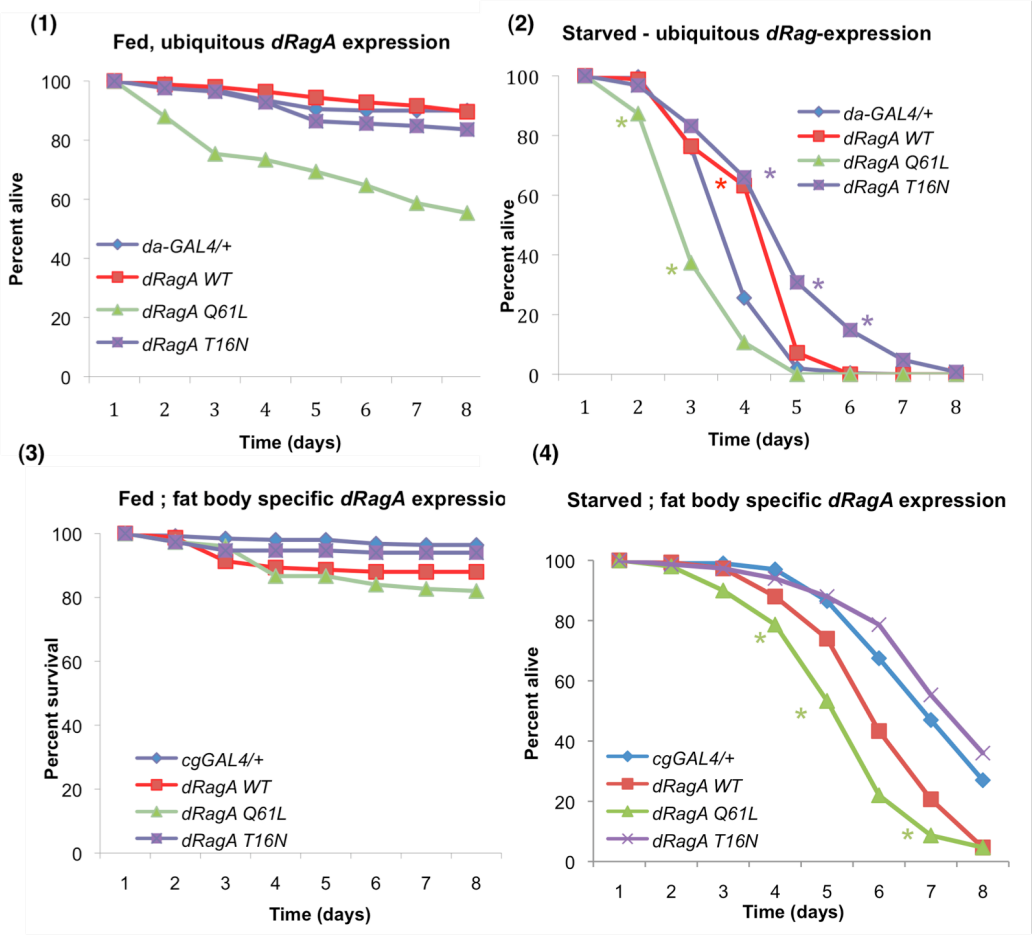


Figure 4H: dRagA activity increases sensitivity to death by starvation.

Ubiquitous (2) or fat body specific (4) expression of dRagAQ61L increases the death rate of adult female flies on complete starvation in comparison to control adult females expressing the appropriate GAL4 drivers (1,3). Expression of dRagAT16N ubiquitously (2) or in a fat body specific manner (4) results in reduced sensitivity to starvation observed as increased viability. Significant difference as compared to control adult viability rate are marked by an asterisk at the corresponding time point (P values < 0.05, Student's 2-tailed t-test)

VII. Relationship between Rheb and dRag-complex

We identified dRag GTPases as positive regulators of TOR activity. Similar to Rheb GTPase, this complex functions to activate cell growth in a S6K and 4EBP dependent manner.

Based on genetic interactions with Tsc1[29], we concluded that dRag complex promotes TOR activity either downstream or in parallel with the TSC-complex. *Drosophila* larvae carrying the P-insertional mutant of *dRagC* die as early third-instar larvae, similar to the lethality seen in *TOR* null mutants. We carried out genetic interactions to test if the P-insertional mutant of *dRagC* can change the lethality of *TOR* null mutant animals. If *dRagC* were downstream to *TOR* then the double mutants would be expected to die at an earlier stage, which was not observed (data not shown). We also carried out genetic interactions between Rheb and the P-insertional mutant of *dRagC*, by checking if *dRagC* can alter the overgrowth phenotype of eye specific Rheb overexpression. No difference in the overgrown eye phenotype was observed (data not shown).

Since our experiments show that dRag complex is important for nutrient sensing, we decided to investigate if dRag activity was needed for Rheb mediated overgrowth observed in the fat body on amino acid starvation. Rheb overexpression in the fat body results in increased cell size (Saucedo LJ et al., 2003). The effect of Rheb overexpression in *dRagC* mutant cells was examined and not found to be inhibitory to the Rheb-induced overgrowth. Interestingly,

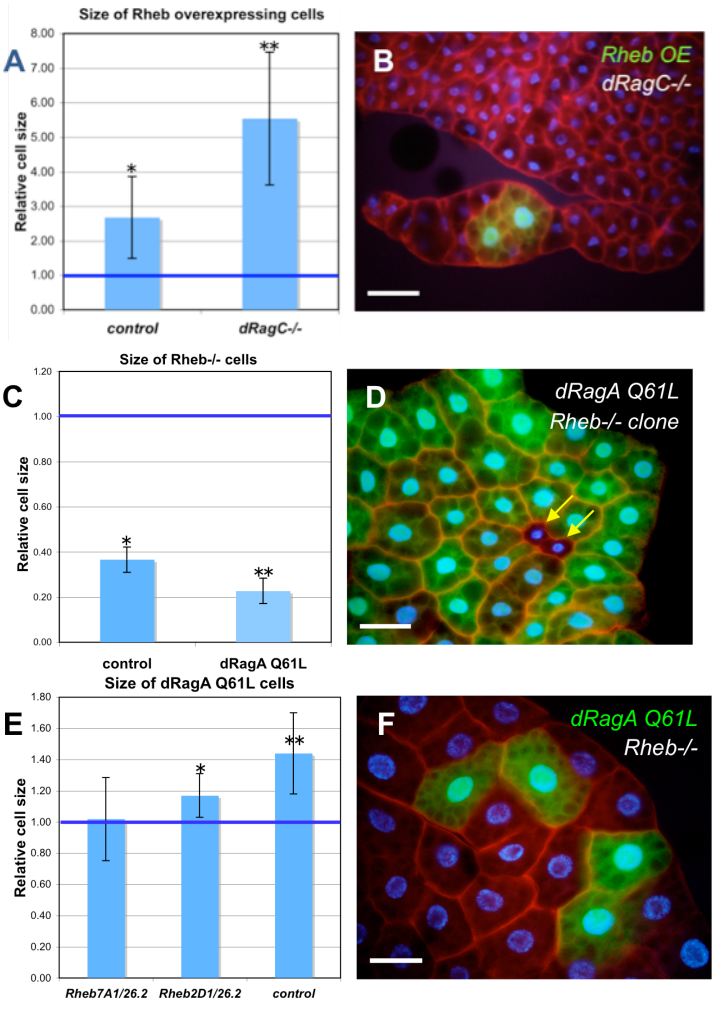
dRagC mutant background induced a more dramatic Rheb growth stimulatory effect than the control wild type background [Fig. 4I-A, B]. Fat body cells with Rheb overexpression are significantly increased in size as compared to the surrounding cells in a *dRagC* homozygous mutant animal. This is similar to the observation of highly increased cell size on Rheb overexpression on starvation, suggesting that nutrient signaling is impaired in *dRagC* mutant animals.

We also tested the effect of constitutive activation of dRagA on Rheb loss of function mitotic clones in the fat body. Rheb mutant cells are smaller than surrounding wild type cells in both control and ubiquitously *dRagA Q61L* expressing larvae [Fig. 4I-C, D] These data suggest that dRag complex is not required for Rheb growth stimulatory effects and acts in parallel to activate TOR pathway.

Conversely, we also analyzed whether Rheb is needed for dRag growth stimulatory effects in the fat body. Clonal expression of constitutively active *dRagA* increased cell size in both wild type and *Rheb* hypomorphic mutant animals, although the effect in *Rheb* mutants was less dramatic [Fig. 4I-E, F]. This suggests that Rheb activity may be required for growth effects of the dRag complex. Taken together, our results indicate that dRag GTPases function to promote growth and cell size either in parallel with or upstream of Rheb.

Fig. 4I: dRags act in parallel with Rheb to promote growth. (A, B) dRags are not required for Rheb mediated growth stimulation. (A) Fat body cells overexpressing Rheb clonally are significantly increased in size both in a dRagC homozygous mutant and control background, relative to surrounding cells (assigned value of 1.0 shown by line). Data are mean ± s.d.; P values = *0.034, **6.1 x 10^{-3} (n=5 where n is number of tested samples under fed condition), Student's 2-tailed t-test. (B) A representative image with Rheb expression marked by GFP, cell boundary marked by Phalloidin (red) and nuclei marked by DAPI (blue), scale bar = 50 μm. (C, D) dRagA Q61L expression cannot rescue reduced cell size in Rheb loss of function mitotic clones. (C). Clonally induced Rheb homozygous mutants (marked by loss of GFP expression in D) are significantly smaller in size relative to surrounding cells in both control and larvae ubiquitously expressing dRagAQ61L. Data are mean ± s.d.; P values = *2.91 x 10^{-4} (n=7), **2.59 x 10^{-8} (n=5 where n=number of tested samples under fed condition), Student's 2-tailed t-test.

(E, F). dRagAQ61L stimulated growth is partially dependent on Rheb. (E) Clonal expression of dRagAQ61L causes a significant increase in control and $Rheb^{2D1/26.2}$ larvae but not in $Rheb^{7A1/26.2}$ animals relative to surrounding heterozygous cells. Data are mean ± s.d.; P values = *0.01 (n=28), **3.63 x 10^{-85} (n=25 where n=number of tested samples under fed condition), Student's 2-tailed t-test. (F) A representative image of dRagQ61L expression in $Rheb^{2D1/26.2}$ hypomorphic background, where GFP co-expression marks dRagAQ61L expression.



VIII. Summary

In both mammalian and yeast models, Rag GTPases function in processes that overlap with TOR activity. Using a reverse genetics approach, we identified Rag GTPases as potential novel upregulators of TOR pathway. In both mammalian and yeast models, Rag GTPases function in processes that overlap with TOR activity. In confirmation of the same, dRags were found to genetically interact with TSC complex, a negative regulator of TOR pathway [Fig. 4A]. dRags were also identified as important for TOR activity as measured by phosphorylation of two downstream effectors for translation, S6K and 4EBP, in S2 cells (Kim E et al., 2008).

Based on its homology with yeast and mammalian Rag genes, we speculated that the functional dRag complex consists of dRagA and dRagC though they may be in different nucleotide bound form. The dRag complex contribution towards TOR signaling *in vivo* was therefore tested using mutants in either dRagA or dRagC.

Our data suggest that dRag-GTPases positively contribute to enhance growth and cell size via upregulation of TORC1. Enhancing dRag activity increased growth of the wing compartment as well as individual wing cell size where it was expressed [Fig. 4C,D]. Compromising dRag activity under nutrient sufficiency lead to decreased cell size in *Drosophila* fat body, a key tissue for nutrient sensing [Fig. 4F]. Interestingly, cells with hyperactivated dRag complex

that were starved for amino acids for 48 hours underwent a false sense of nutrient sufficiency [Fig 4F]. These fat body cells expressing highly active dRagA were unable to respond normally to starvation since they failed to induce autophagy [Fig. 4G], a catabolic process that normally aids nutritional provision under starvation. Activation of dRag in adult flies, by expressing constitutively active dRagA, mimicked nutrient sufficiency under complete starvation. As a result these adult flies failed to switch their metabolism from an active physiological state to a resting or maintenance state, leading to an increased susceptibility to death by starvation [Fig. 4H].

Our genetic interaction studies point to dRags acting in parallel or upstream of Rheb to activate TOR signaling [Fig. 4I]. All the above results indicate that dRags are important for nutrient sensing and are the missing link for amino acid sensing by the TOR pathway. The details of the same and a model of their possible mechanistic action will be addressed further in Chapter 5.

CHAPTER 5. A POSSIBLE MECHANISM FOR dRag-MEDIATED AMINO ACID REGULATION OF TORC1

I. Existing models for dRag-mediated amino acid sensing by TOR pathway

Our model [Fig. 5A] based on studies with *Drosophila* and mammalian Rags suggest that Rags activate TORC1 indirectly, possibly through affecting TOR localization or by affecting another unknown direct regulator of TOR. Though Rags sense amino acids, they do not mediate direct uptake of amino acids as seen with cell culture experiments carried out by E.Kim. In a parallel and independent study, Sancak Y. et al, identified Rags as direct interactors of Raptor, a TORC1 component. Their model [Sancak Y et al., 2008] based on studies with mTOR antibody, show that indeed amino acid sensing through Rags consequently changes mTORC1 localization from cytosolic punctae to a subcellular endosomal localization enriched with Rheb and Rab5 vesicles. However, how this localization or movement of TORC1 is brought about still remains unclear. It is intriguing to speculate that another novel regulator may be involved that brings about this translocation.

One interesting candidate that has come out of the RNAi screen from the K.L Guan's group is Arf1. Depletion of Arf1 in *Drosophila* S2 cells using RNAi decreased TORC1 activity as measured by S6K and 4EBP phosphorylation (unpublished data).

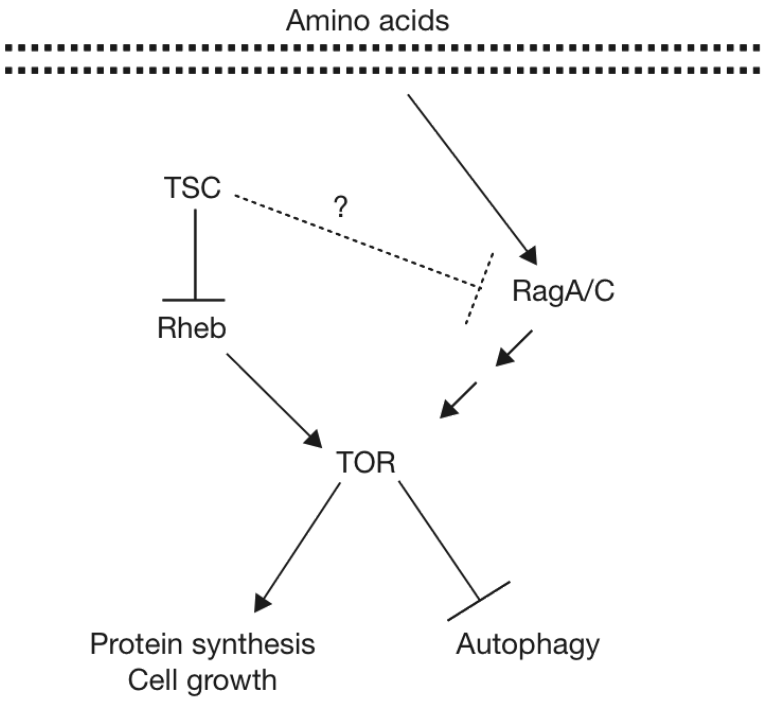


Fig 5A: Proposed model for dRag regulation of TORC1 activity

Rag GTPases act either independently or upstream of Rheb to activate TOR signaling. Our genetic interactions with Tsc1 indicate that Rag-GTPases may be either downstream or in parallel to the TSC-Rheb axis of TOR pathway. Cell culture studies in Kim. E et al, suggest that Rag GTPases do not promote availability of amino acids by direct uptake. *dRag* regulation of TORC1 is indirect, possibly through involvement of unknown players and may be due to Rag regulation of TORC1 via regulating its compartmentalization.

II. Arf-GTPases and their biological significance

ADP-ribosylation factor (Arf) proteins are evolutionary conserved proteins belonging to the Ras-family of small GTPases. The activity of Arf GTPases was first identified as important for toxicity of the bacterial cholera toxin to ADP-ribosylate heterotrimeric G-protein (Kahn RA et al., 1986). Subsequently six Arf-GTPases that fall into three different classes based on their amino acid sequence homology were identified. *Drosophila* has one representative member of each Class of Arf GTPases, namely Arf79F, Arf102F and Arf51F that are homologous to the mammalian Class 1, 2 and 3 respectively (Lee FS et al., 1994, Gillingham and Munro, 2007). The most well known function of Arf-GTPases is their conserved role in membrane trafficking, understood particularly through studies with Arf1. Arf1-mediated actin dynamics regulates vesicle trafficking at the Golgi by recruiting effectors such as vesicular coat proteins (COPI), clathrin adaptor protein 1 (AP1) and the Golgi localized γ-ear containing Arf binding proteins (GGAs) (D'Souza-Schorey and Chavrier, 2006). The ability of Arf1 to recruit its binding effectors is dependent on the minor structural changes induced on GTP loading of Arf1 such as generating membrane curvature and on its subcellular localization (Lundmark R et al., 2008). Mammalian Arf GTPases, in addition to Rheb and Rho GTPases, bind to and activate Phospholipase D (PLD*) in vitro* (Cockcroft S et al., 1984, Powner and Wakelam, 2002, Sun Y et al., 2008). There is no consensus about which of these different GTPases is significant for PLD activation *in vivo*. Recently, in an *in vitro* reconstituted HeLa cell system, where

Arf1 was bound to liposomes with a defined lipid composition, the resulting Arf1-mediated actin dynamics was shown to produce movement of the liposome (Heuvingh J et al., 2007). This finding is very interesting as it supports a role for Arf1 as a candidate for causing TORC1 translocation in response to an appropriate activating signal.

It is possible that Arf-GTPases can regulate TORC1 activity in more than one way. It has been shown that *in vitro*, both Rheb and Arf1 activate PLD. Activated PLD generates higher amounts of PA, which has been shown to directly bind to and activate TORC1 (Fang Y et al., 2001). Most interestingly, TCTP/p23, the now questionable Rheb–GEF, functions as a receptor for Arf1 at the Golgi before guanyl nucleotide exchange occurs (Gommel DU et al., 2001, Gatchett Y et al., 1999). Using a cross-link approach it was demonstrated that the cytoplasmic tail of TCTP directly binds to a 22-amino acid domain at the C-terminal of GDP bound Arf1 thereby recruiting it to the Golgi for initiating downstream effects for vesicle trafficking and actin dynamics. This may explain the inconsistency in TCTP studies, especially if TCTP functions as a GEF not for Rheb but for Arf1 to initiate actin dynamics that eventually result in translocation of either TORC1 or of some of the complex members that brings it in proximity to Rheb.

All of the above data points to an intriguing role played by Arf1 for TORC1 activation. As a follow-up to our studies on Rag GTPases, we decided to

investigate whether Arf1 acts as a positive regulator for TOR activity *in vivo* and to test whether it can cause subcellular translocation of TOR between nutrient sufficiency and nutrient limitation.

III. *Arf79F* functions as a positive regulator of TORC1

The *Drosophila* homolog of *Arf1*, *Arf79F*, is present at the base of the left arm of the third chromosome. This chromosomal location made recombination of *Arf1* with *FRT80B* extremely difficult, which would normally be ideal for analyzing loss-of-function clonal effects of Arf1. Instead, we decided to use a UAS-ARf79FRNAi (referred to henceforth as Arf79FRNAi) transgenic stock from the Vienna Stock Center that would enable us to study effects of Arf79F knockdown.

TORC1 activity is important for regulating cell size and growth. In order to analyze effects of inactivated Arf79F on TORC1 activity, we looked at fat body cell size in well-fed third instar larvae using Phalloidin assay. The Arf79FRNAi stock is viable by itself and hence in order to strengthen the RNAi effect, we expressed Dicer along with ARf79FRNAi. Under nutrient sufficiency conditions, knock down of Arf79F results in a reduction of cell size [Fig5B] while the control cells that are wildtype FLP-out clones do not exhibit any effects of cell size. Another specific downstream function of TORC1 is inhibition of starvation-induced autophagy under nutrient sufficiency conditions. We looked at the effects of Arf79F knockdown on autophagy using Lysotracker assay in fat body cells of third instar larvae that expressed both Dicer and Arf79FRNAi. Reduced activity of

Arf79F induced autophagy in the well-fed state [Fig5C]. Together, these data support the hypothesis that Arf79F may act as a positive regulator of TORC1 signaling *in vivo*, consistent with the effects of Arf79F RNAi on TOR-dependent phosphorylation of S6K and 4EBP observed by our collaborators.

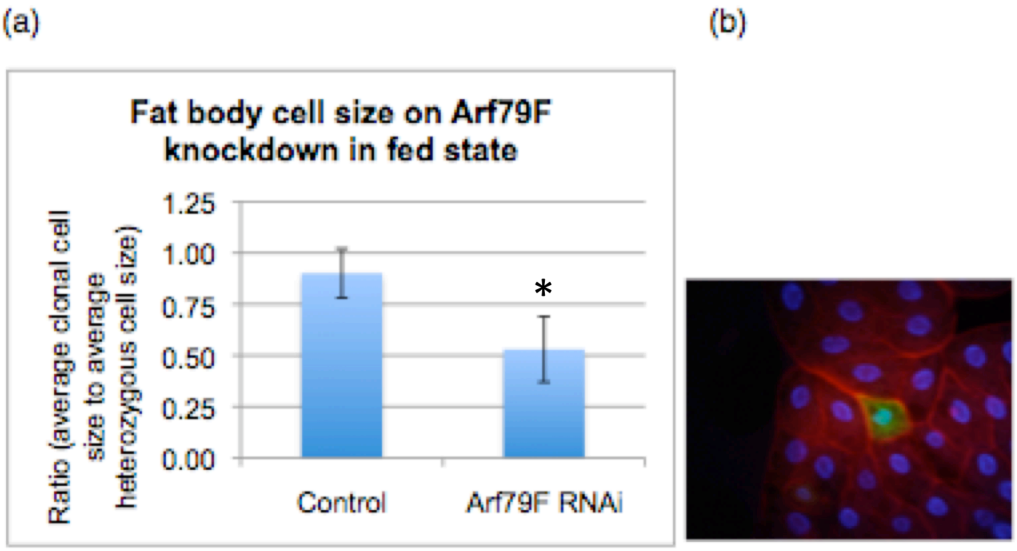


Fig 5B. Arf79F positively regulates fat body cell size under nutrient sufficiency

Phalloidin assay was carried out on fat body cells from larvae that were expressing Arf79RNAi clonally using the Act>CD2>UASGFP,GAL4 under well fed state. Knockdown of Arf79F decreases ratio of cell size significantly as compared to cells from well fed larvae expressing just Act>CD2>UASGFP,GAL4 (a). A representative image is shown where GFP marks UAS-Arf79FRNAi expression, red represents phalloidin marking actin at the cell boundary and blue (DAPI) represents nuclei. Data are mean ± S.D.; P vaues: ∗ 2.7 x 10^{-5} (number of larvae = 25), Student's 2 tailed t-test.

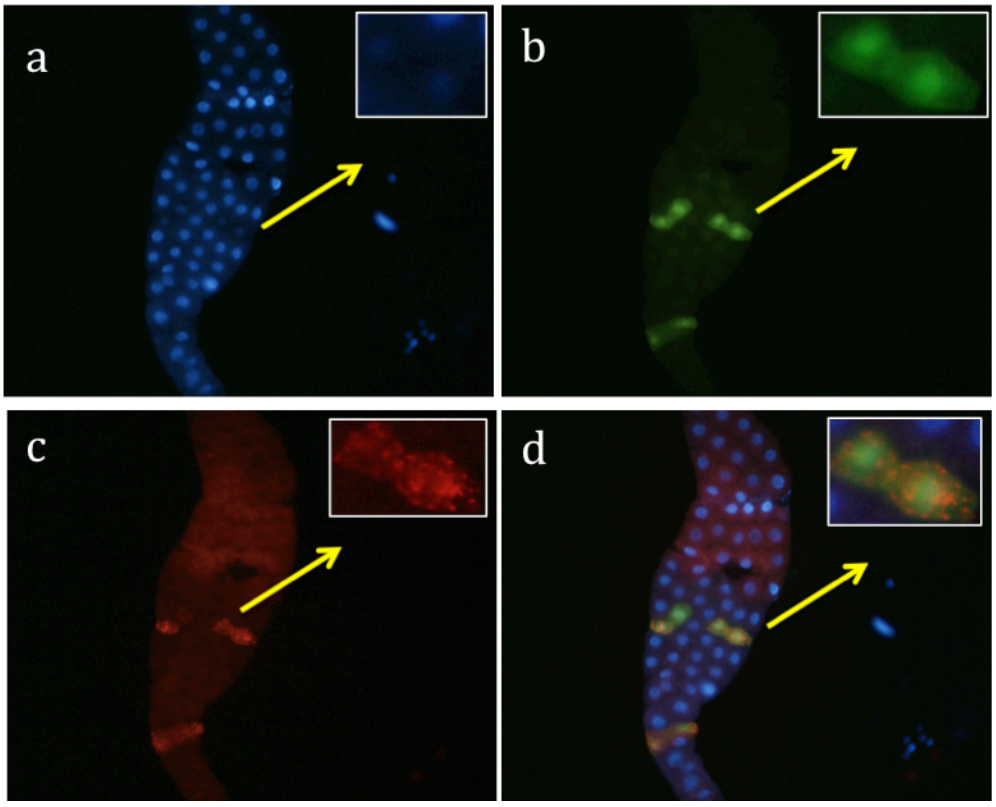


Fig 5C: Arf79F knockdown results in appropriate induction of autophagy under nutrient sufficiency

Autophagy is induced in fat body cells of well fed early third instar larvae expressing *Arf79RNAi* clonally using Act>CD2>UASGFP,GAL4 along with UAS-Dicer. The GFP positive cells which have Arf79F knocked down inappropriately induce autophagy as seen with incorporation of the red LysoTracker dye which marks the expanded autolysosomal compartment. Panel a shows DAPI marking the nucleus, panel b shows Arf79RNAi expressing clonal cells in green and red punctae indicate autolysosomal compartments with acidic pH. A representative set of clonal cells is enlarged in the box in each panel. Number of larvae tested=32

III. Role of Arf79F in mediating TOR localization

A recent study showed that it is possible to cause movement of Arf1-tagged liposomes in HeLa cells *in vitro*, via the Arf1 generated actin dynamics. This led us to speculate that Arf79F could be involved in translocation of TOR from an active state, seen on amino acid sufficiency, to a subcellular localization seen on amino acid limitation. Unlike the mammalian system, we do not have a good antibody that recognizes *Drosophila* TOR. Instead, we decided to use antiFLAG antibody to study localization of overexpressed FLAG-TOR and analyze how it varies on reducing Arf79F activity clonally. FLAG-TOR expression was induced by one-hour 37°C heat shock, 48 hours AEL. *Arf79FRNAi* expression was induced in fat body cells of third instar larvae that were either well fed or were starved for amino acids for 4 hours on 20% sucrose using the FLP-out GAL4 technique and FLAG-TOR localization pattern was analyzed 48 hours post-heat shock induction.

As a control, we looked at larvae that co-expressed FLAG-TOR and the Act>CD2>Gal4, UASGFP without Arf79FRNAi expression, in both well-fed and amino acid starved state. Similar to previous studies with FLAG-TOR in the lab (Hennig KM, Neufeld TP), it was noted that FLAG-TOR localization is variable to some extent despite a tightly controlled induction time point, possibly due to interference with endogenous TOR localization. We therefore analyzed at least

25 animals per genotype and quantified the observed expression patterns as noted below.

In control well fed larvae, localization of FLAG-TOR is mostly cytoplasmic with more FLAG-punctae at the cell membrane rather than in the cytoplasm [Fig 5Da-c]. Fat body cells from control larvae that were starved for amino acids on 20% sucrose for 4 hours (conditions of TOR inactivation) exhibited a very striking pattern. In 66% of these cells, FLAG localization is in mostly nuclear fashion with one bright spot along the nuclear periphery [Fig 5Dd-f]. In fat body cells that were overexpressing Arf79FRNAi and FLAG-TOR during the fed state, 72% cells exhibited an increased number of cytoplasmic punctae versus punctae at cell membrane, 16% of the cells exhibited nuclear TOR, which is inactive [FigDg-i], while rest of the 13% cells exhibited diffused cytoplasmic localization. This suggests that Arf79F inactivation under nutrient sufficiency conditions fails to shift TOR to a cytoplasmic localization normally observed with nutrient sufficiency pattern. In starved larvae that co-expressed Arf79FRNAi and FLAG-TOR, TOR localization was not always cytoplasmic or at the cell membrane. 64% of the cells exhibited a ubiquitous diffused FLAG staining while 36% exhibited nuclear FLAG staining [Fig5Dj-l].

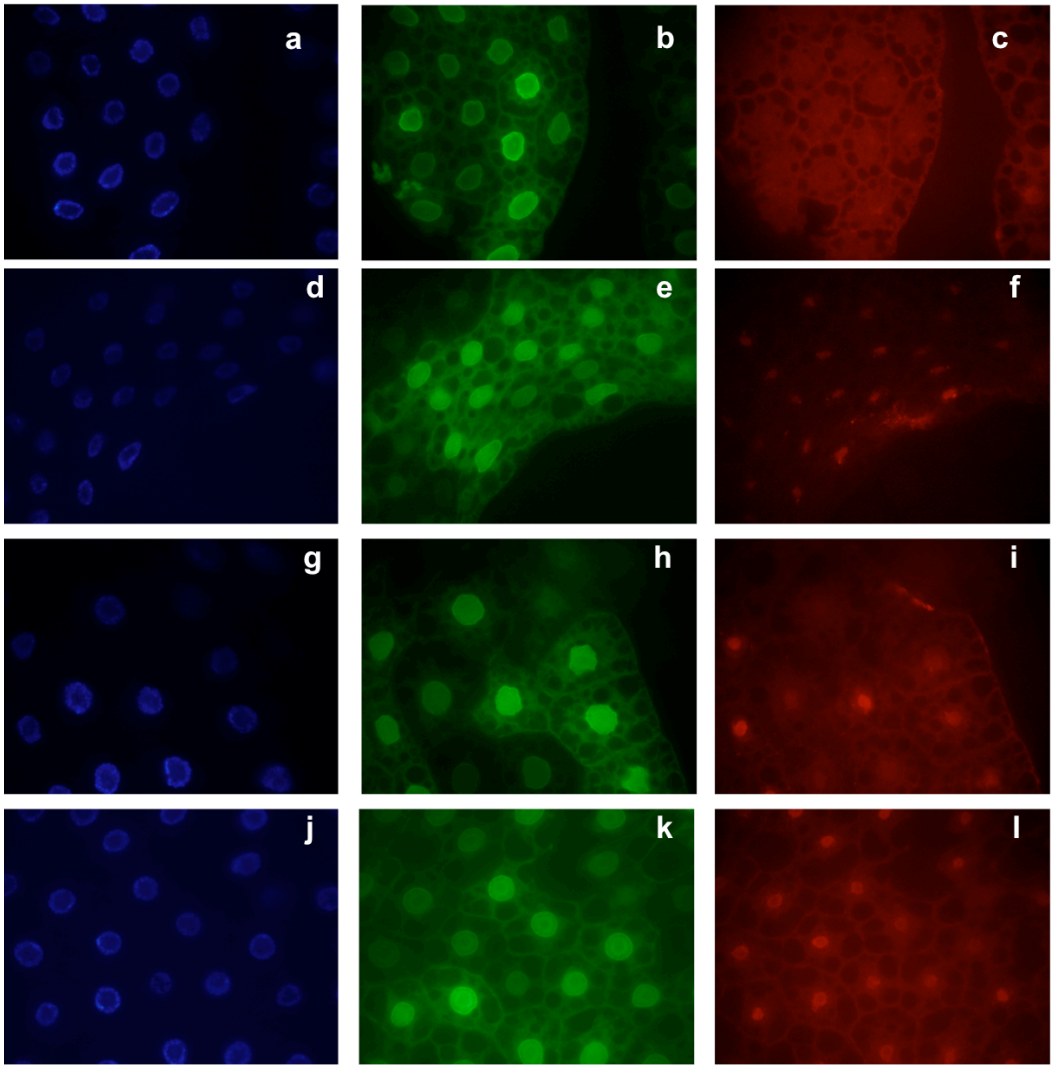


Fig 5D: Arf79F is required for localization of TOR to an active compartment that is cytoplasmic and cell membranous.

In wildtype control larvae with only Act>CD2>UASGFPGAL4, under nutrient sufficiency, FLAG-TOR is active (in red) and FLAG exhibits a diffused cytosolic and cell membranous punctal location (a-c) in fat body cells. On amino acid starvation for 4 hours, FLAG-TOR goes into the nucleus, which denotes inactive TOR location. Expression of Arf79FRNAi clonally, marked by GFP positive cells, in larval fat body on amino acid limitation does not change inactive TOR localization pattern (j-l). Expression of Arf79FRNAi clonally, as marked by GFP positive cells, in larval fat body under nutrient sufficiency, exhibits inactive TOR localization. Red represents antiFLAG antibody and Blue represents nucleus as marked by Hoechst. Number of larvae tested for each genotype ≥ 25

Based on our previous results, we know that highly active RagA results in hyperactive TOR activity, especially on amino acid limitation. All the fat body cells that co-expressed constitutively active RagA and FLAG-TOR exhibited a diffused cytoplasmic pattern of FLAG in the well-fed state [Fig5Ea-c]. Most interestingly, amongst cells from larvae that co-expressed constitutively active RagA and FLAG-TOR and underwent amino acid starvation for 4 hours, 100% exhibited the observed nutrient sufficiency TOR localization pattern with 62% FLAG punctae at cell membrane and 38% with diffused cytoplasmic FLAG pattern while most of the internal control cells that did not express constitutively active RagA exhibited nuclear FLAG-TOR [Fig5Ed-f], as expected.

In order to determine if Arf79F was required for TOR activation on amino acid availability, we tested whether FLAG-TOR localization shifts in cells that overexpressed constitutively active RagA and FLAG-TOR in Arf79FRNAi expressing background. In well-fed larvae that co-expressed Arf79FRNAi, FLAG-TOR and constitutively active RagA, 14% cells exhibited FLAG pattern similar in the starvation nuclear pattern while rest of the cells showed a diffused cytoplasmic pattern or FLAG punctae at the cell membrane [Fig5Eg-i]. Regardless, since we never see nuclear FLAG-TOR localization in fed state in any genetic background unless we knock down Arf79F levels, the above observation suggests that Arf79F activity is important and required for causing translocation of TOR to an active state.

Fat body cells from larvae that were starved and co-expressed Arf79FRNAi, FLAG-TOR, and constitutively active RagA exhibited the starvation FLAG-TOR localization in a nuclear pattern 52% times [Fig 5Ej-l] or a diffused ubiquitous cytoplasmic localization 48% times instead of the active FLAG punctae at cell membrane. Thus while overexpression of constitutively active RagA on amino acid starvation mimics amino acid availability, knockdown of Arf79F in this background can override the observed TOR activation [Fig 5F]. This suggests that Arf79F is involved in translocating TOR or possibly TORC1 in between two different subcellular localizations depending upon availability of amino acid.

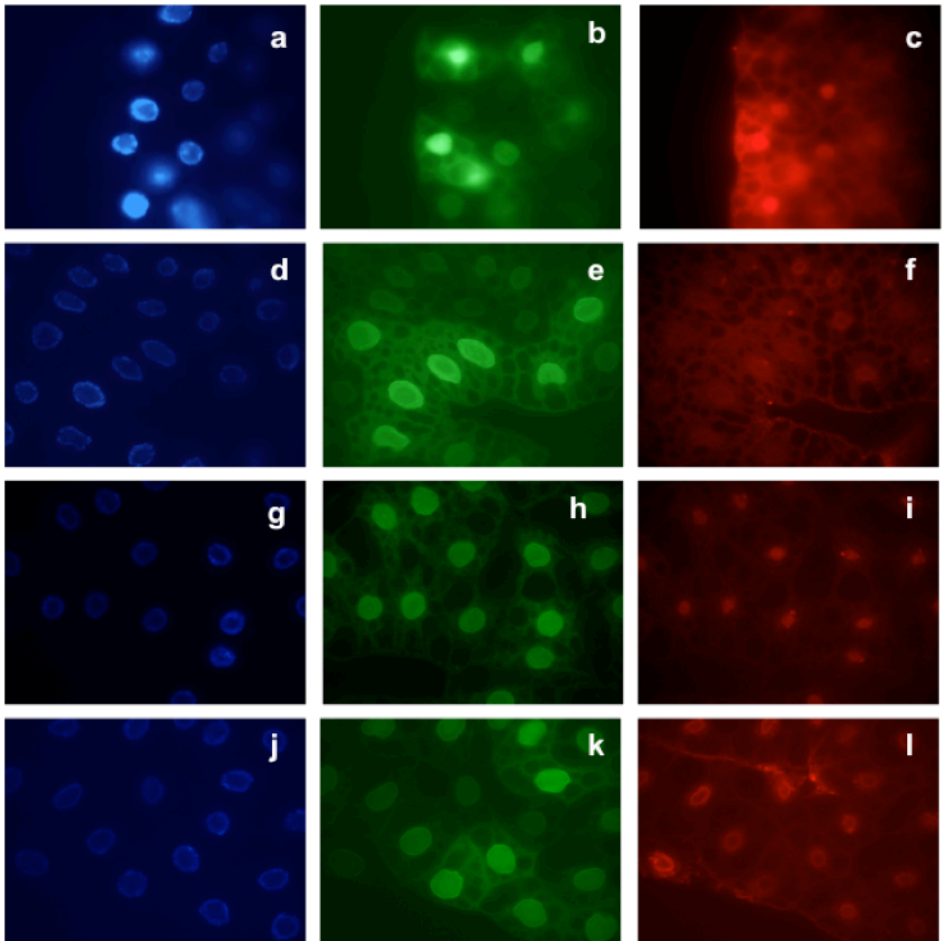


Fig 5E: Knockdown of Arf79F inhibits TOR hyperactivation normally seen in constitutively active *dRagA* expressing fat body cells under nutrient sufficiency

Clonal overexpression of *dRagAQ61L* as marked by GFP expressing cells in well-fed larval fat body using FLP-out GAL4 results in hyperactive TOR, which exists in a diffused subcellular localization (a-c). Even after 4-hour starvation, *dRagAQ61*L expressing cells, marked by GFP co-expression, cannot switch to the starvation nuclear TOR pattern (d-f) while some of the surrounding heterozygous cells, not marked by GFP, exhibit the nuclear TOR pattern. Co-overexpression of Arf79FRNAi and *dRagAQ61L* in fat body cells, marked by GFP expression, exhibits the starvation nuclear pattern of TOR localization with hardly any cytoplasmic FLAG signal, even under nutrient sufficiency (g-h). TOR localization is not affected in cells co-expressing *Arf79FRNAi* and *dRagQ61L* under amino acid limitation (j-l). In all above cells, blue represents nuclei by Hoechst staining and red represents anti-FLAG antibody, which marks FLAG-TOR expression. Number of larvae tested for each genotype ≥ 25

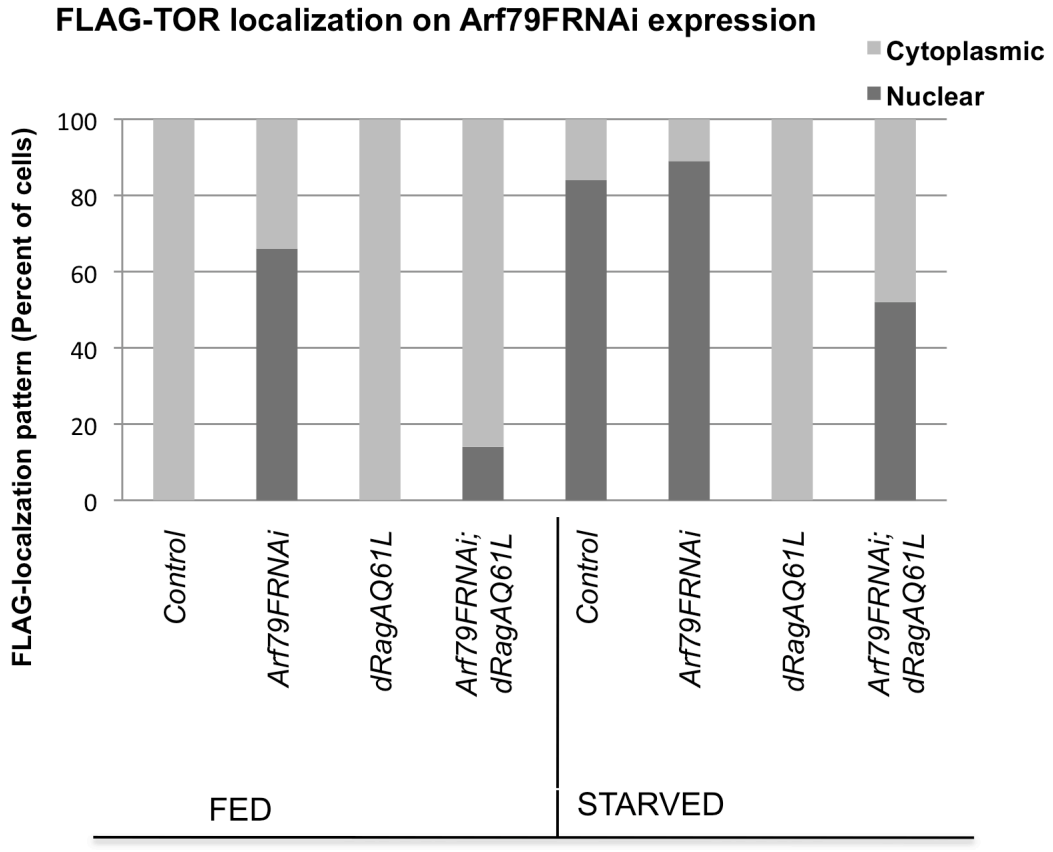


Fig 5F. Summary of FLAG-TOR localization seen in different genetic backgrounds

In wildtype control larval fat body cells, FLAG-TOR localizes to the cytoplasm and the nucleus under nutrient sufficiency and nutrient limitation respectively. In well-fed condition where TOR is generally active, inhibiting Arf79F activity results in TOR localization pattern reminiscent of starvation (in the nucleus). As expected with previous observations, dRagAQ61L expression results in TOR activation as seen with cytoplasmic FLAG-TOR localization pattern in both fed and starved condition. Inactivating Arf79F inhibits well-fed cytoplasmic localization of FLAG-TOR on dRagAQ61L expression in both starved and fed conditions suggesting that Arf79F is required for TOR activation by dRags.

IV. Summary

It is now speculated that in addition to its kinase activity and association with effector proteins, TOR localization is important in regulating growth in part by affecting subcellular proximity of TOR to its downstream and upstream effectors. In line with this idea, a recent model predicts that on sensing amino acids by Rag GTPases, TORC1 translocates from an inactive cytoplasmic punctal localization to an endosomal compartment that brings it in close proximity to its upstream regulator Rheb for further activation (Sancak Y et al., 2008). How exactly this translocation is mediated is not clear but its significance for downstream TOR activity is crucial.

The *Drosophila* Arf1, Arf79F, was identified as a novel positive regulator of TORC1 activity in a S2 cell based RNAi screen, as measured by decreased phosphorylation of two key downstream TORC1 targets, namely S6K and 4EBP (unpublished data). Arf-GTPases are evolutionary conserved GTPases that mediate growth via regulation of membrane trafficking and actin dynamics at the Golgi. Most interestingly, Arf-GTPases are conserved in the protist *Giardia lamblia*, which lacks heterotrimeric G proteins and small GTPases (Murtagh JJ et al 1992, Li Y et al, 2004). This points out that both TOR and Arfs are ancient players, and given our knowledge of the evolutionary conserved multiple functions governed by TOR for spatial and temporal aspects of growth, it is not surprising that Arf1 can regulate TOR activity.

Our data attests that Arf79F positively regulates TOR-mediated growth and cell size [Fig.5B,D]. Similar to other TOR positive regulators, inactivating Arf79F induces autophagy inappropriately under nutrient sufficiency [Fig.5C]. Due to recent reports that Arf1-mediated actin dynamics can cause movement of liposomes *in vitro*, we tested Arf79F as a potential candidate for mediating translocation of TOR on amino acid starvation. Unfortunately, we are limited in availability of a good antibody for *Drosophila* TOR protein. Regardless, we used anti-FLAG antibody to analyze FLAG-TOR localization under conditions of nutrient sufficiency and limitation in different genetic backgrounds. We observe that, indeed, Arf79F knockdown can block TOR activation as measured by TOR localization pattern in a constitutively active dRagA expressing background, which normally mimics a false sense of nutrient sufficiency to the cells [Fig. 5E,F]. This implies that Arf79F is required for dRag-mediated TORC1 activation.

Whether Arf79F overexpression can overcome the effects of dominant-negative *dRagA* overexpression or growth defects in endogenous *dRagC* mutants, which normally arise from reduced TOR-mediated growth and cell size, remains to be tested. Since Arf1 interacts with TCTP, a previous contender for positive regulation of TOR pathway via its GEF activity, and activates PLD *in vitro* to increase levels of PA, another positive regulator of TOR, it is important to analyze how these events affect Arf79F-mediated TOR translocation. It is possible that a number of these events additively enhance overall TOR activity.

At present, we have not tested for any overlap between Arf1 and TORC2 mediated actin dynamics, but it also remains a possibility. It is also unclear whether Arf1 causes TOR regulation through its GTPase activity, vesicular trafficking or actin polymerization. The detailed analyses of Arf1-mediated positive regulation of TORC1 await further characterization.

CHAPTER 6: DISCUSSION

The evolutionary conserved TOR pathway plays a crucial role in maintaining global and cellular growth homeostasis with respect to availability of amino acids and growth factors, and cellular energy and stress levels. Recently it is found that TOR regulates temporal and spatial aspects of cellular growth in multiple ways via existing in two complexes, TORC1 and TORC2, and on integrating various signals. The TOR pathway has been dissected using elegant genetic and biochemical analyses leading to identification of the upstream regulators and downstream effectors. However, interplay of TOR signaling with a number of diverse signals (Figure 1A) is emerging, opening up the possibility of new TOR downstream effectors as well as novel regulators. Our study successfully addressed the identification and characterization of such regulators or effectors that are imperative for enhancing TOR activity using forward and reverse genetic approaches.

I. Amino acid sensing

The importance of amino acid regulation of TOR-mediated growth is well known. Amino acid withdrawal not only hinders TOR activity as measured by S6K and 4EBP-phosphorylation but also makes it unresponsive to insulin stimulation. The exact regulator that is responsible for sensing amino acids and modulating TOR activity accordingly has been unknown for about two decades. The most interesting candidate identified thus far was hVps34 (Nobukini T et al., 2005). In

mammalian cell culture, hVps34 lipid kinase activity is regulated by amino acids via increasing intracellular levels of $Ca2^{+}$ (Gulati P et al., 2008). However, in spite of its importance for growth via its role in endocytosis, there has been no validation of its role in amino acid sensing *in vivo* in non-mammalian systems such as *Drosophila* and *C. elegans*. This is surprising since amino acid mediated growth control achieved by TOR is by far the most ancient aspect of this key pathway. Most importantly, the importance of Vps34 for macroautophagy via binding to Beclin, mammalian homolog of Atg6 (Furuya N et al., 2005), is well demonstrated. Thus it is difficult to reconcile how Vps34 can stimulate both autophagy and TORC1 activation, which normally inhibits autophagy on amino acid sensing. It is possible that by existing in two different complexes that co-localize to different intracellular locations, Vps34 can achieve two opposing outputs. It is also possible that Vps34 lipid activity generates PI3P, which then participates in stimulating TORC1 activity possibly by acting as a signaling platform at the endosomal membrane. Indeed, sequestering PI3P by overexpressing FVYE domain hinders S6K phosphorylation (Nobukini T et al., 2005). Nevertheless, the role of Vps34 in nutrient-mediated TORc1 signaling remains to be established in detail to address the above questions and the conservation of its activity in different model systems both *in vivo* and *in vitro*.

Our study, in collaboration with studies in K.L Guan's lab has identified that Rag GTPases function for amino acid sensing and therefore regulate TORC1

activity. Rag GTPases are evolutionary conserved from yeast to higher eukaryotes. Independently and in parallel with David Sabatini's group, we have shown that Rag GTPases mediate amino acid sensing in mammals and *Drosophila* (Kim E et al., 2008 and Sancak Y et al., 2008). Previous studies in yeast have already demonstrated that the yeast Rag GTPases (Gtr1 and Gtr2), as a part of the EGO/GSE complex, are important for TOR-mediated GAP1 (General amino acid permease 1) translocation to the cell membrane under amino acid limiting condition. Similar to the yeast Class C *vps* mutants, *gtr1/2* exhibit synthetic lethality with *tor1* mutants (Doubouloz et al., 2005, Zurita-Mertinez et al., 2007). Our finding is conserved in yeast, *Drosophila,* and mammalian systems as expected of the most ancient amino acid sensing function in eukaryotes, both *in vivo* and *in vitro*.

II. Rag-GTPase activity and function in nutrient response

In our study, using siRNA screening in *Drosophila* S2 cells in K.L Guan's lab and using our reverse genetics approach, we have identified dRag GTPases (dRagA and dRagC) to be important for nutrient mediated TORC1 signaling. Endogenous *dRag* complex acts as a bonafide positive regulator of TORC1 by virtue of the ability of *dRagC* mutation to dominantly rescue early larval lethality observed in $Tsc1^{29}$ null mutants. Compromising the dRag-complex activity, by inhibiting endogenous dRagC or overexpressing dominant negative dRagA, results in decreased fat body cell size only under amino acid sufficiency. On

amino acid starvation, overexpression of constitutively active *dRagA* is sufficient to increase fat body cell size while inhibiting starvation-induced autophagy. This clearly points to the involvement of dRags in nutrient-mediated TOR induced growth effects. Studies with mammalian cells show that the growth stimulatory effects of constitutively active RagA specifically prevail over amino acid starvation and not osmotic stress (E. Kim et al., 2008). Also, though GTP-bound RagA/B activity can substitute for lack of amino acids, it cannot replace insulin signal pointing to the key involvement of Rags in amino acid sensing. Data from both *Drosophila in vivo* studies and cell culture experiments in mammalian and S2 cells prove that Rags primarily affect TORC1 mediated translational growth and not TORC2 mediated spatial growth. The importance of dRags in regulating nutrient response is also seen by the inability of constitutively active *dRagA* expressing fat body clonal cells to induce starvation-induced autophagy [Fig 4G] and the resulting modulation of global metabolism in *dRag* expressing adults. Completely starved flies overexpressing constitutively active *dRagA*, ubiquitously or in a fat body specific manner, die earlier than control flies presumably due to a failure to attenuate their metabolism and growth owing to a false sense of nutrient sufficiency [Fig.4H].

The growth stimulatory effects seen on Rheb-overexpression in *Drosophila* fat body are unaffected by inactivating endogenous dRagC activity [Fig 4I]. In fact, the relative growth stimulation seen on Rheb-overexpression is

much higher in a *dRagC* homozygous mutant animal background than in the control animal background, once again highlighting that nutrient sensing and signaling is impaired on dRag inactivation. Conversely, loss of *Rheb* in fat body cell clones cannot be rescued by overexpression of constitutively active *dRagA*. Interestingly, constitutively active *dRagA* overexpression in the fat body could increase cell size in both wildtype and Rheb hypomorphic background, though not to the same extent. Together, these results suggest that dRag function works either in a parallel pathway to the TSC-Rheb axis or upstream to Rheb for TORC1 activation but that Rheb activity is dominant or needed for TORC1 growth effects.

Biochemical studies carried out with mammalian HEK293 and HeLa cells (by E.Kim et al., 2008) confirmed that Rag GTPases function as a stable heterodimer. Based on mammalian cell culture experiments, RagA/B act as the catalytically active component, active only in the GTP-bound form, while RagC/D function for TORC1-mediated S6K phosphorylation is nucleotide independent. This is also supported by the observation that overexpressing wildtype and constitutively active dRagA versus overexpressing wildtype and constitutively active dRagC increases wing compartmental and cell size. On the other hand, inactivating GTP-binding to dRagC by overexpressing dominant negative dRagC can increase wing compartmental and cell size [Fig 4D]. This is similar to the observation in yeast studies where Gtr1p needs to be in the GTP-bound form

while Gtr2p is GDP-bound to promote Gap1p sorting to the cell membrane instead of vacuole (Gao M & Kasier C, 2005). Nevertheless, RagC/D is important for Rag-complex function as seen with phenotype of *dRagC* mutant animals and effects of dRagC knockdown in S2 cells, perhaps for maintaining stability of the Rag complex, localization and activity of RagA or aiding in TORC1 localization by recruiting binding partners of TOR.

Studies in yeast and mammals have shown that Rags can bind to nuclear proteins such as Ran-GEF, Nop132 and Yrb2p suggesting that nuclear localization may be important for their function (Nakashima N et al., 1999, Sekiguchi T et al, 2003. Yang W et al., 2005). In addition, there is evidence of the yeast Rags, Gtr1 and Gtr2, involvement in microautophagy and GAP1 sorting pointing to an endosomal and vacuolar localization (Dubouloz F et al., 2005, Gao M & Kaiser C, 2006). In light of the endosomal localization of TOR and Rheb being key for TOR downstream activity, this places the Rag GTPases at the right place to stimulate TOR kinase through an unknown or in an indirect manner post-stimulation by amino acids.

Whether the nuclear activities of dRag and TOR overlap with each other remains as a topic for future in depth studies. Both Rags (Gtr1-2) and TOR are implicated in regulation of microautophagy, a process involving direct cytosolic uptake by pinching off the vacuolar membrane. Due to the lack of well-evaluated markers for the lysosomal membrane in *Drosophila*, it was not possible to

address this topic. Thus far there has been no evidence of this process in *Drosophila,* though it is observed in yeast and mammalian liver cells. It will need a number of trials and the generation of an efficient marker to visualize this process in the fly system, and then address the role of TOR pathway for the same.

III. Nature of amino acid sensing

Though we have identified dRags to be important for amino acid sensing, we do not entirely understand the mechanism of how they work to mediate TORC1 upregulation. Studies by E. Kim (unpublished data) show that constitutive active RagA expression does not increase direct import of amino acids, suggesting that Rags do not promote amino acid availability. However, it is not known if RagA/B expression somehow promotes intracellular amino acid increase in starved cells though this is a possibility that remains to be checked. Sancak Y et al, 2008, have provided a working model, which postulates that on sensing amino acid sufficiency, Rag GTPases cause translocation of mTORC1 from cytoplasmic location to a membrane localization shared by endosomal markers Rab7 and Rheb. Thus amino acids induce GTP-charging of RagA, which then binds to mTORC1 component, Raptor, recruiting mTOR in proximity to Rheb. It is not understood how amino acids mediate charging of RagA. One way that this could occur is through increased Ca2+ in light of the observation that addition of branched chain amino acids like leucine cause an increase in the

intracellular Ca2+ levels (Gulati P et al., 2008). Increased Ca2+ levels could subsequently increase GTP production by enhancing 2-oxoglutarate dehydrogenase activity (McCormack and Denton, 1993). If this is the case, the effect of increased levels of Ca2+ should mimic amino acid translocation of mTORC1, which remains to be seen.

As mentioned earlier Rag GTPases themselves have different subcellular locations as well as different binding partners for achieving specific downstream function. Thus it is possible that by existing in different complexes with unique binding partners and in distinct subcellular locations, Rag GTPases themselves may be responsible for causing mTOR or mTORC1 components to move to an active endosomal location. By far, this is the most attractive prospect where Rag complex acts between amino acids and TORC1 via changing localization of TORC1 or its components.

How exactly and what causes the translocation of mTORC1 to the Rab7-enriched compartment is currently unclear. We have investigated the involvement of another novel regulator Arf79F, fly homolog of Arf1 for the same.

IV. Arf79F and its involvement in TOR localization

Hindering Arf79F activity reduces TOR activity as seen with overexpression of UAS-Arf79FRNAi in *Drosophila* larval fat body. Knockdown of Arf79F induces autophagy in larval fat body while reducing fat body cell size [Fig. 5B,C]. A recent *in vitro* study has revealed that Arf1-mediated actin polymerization can result in

movement of artificial lipid vesicles (Heuvingh J et al., 2007). This presents us with the exciting possibility that Arf79F maybe involved in shuttling TOR or TORC1 between different subcellular locations, in response to nutrition. With this speculation, we tested the ability of Arf79F to mediate FLAG-TOR localization in fat body cells under fed, starved, and a false nutrient sufficiency state which leads to hyperactive TOR signaling. We find that indeed inhibiting Arf79F leads to TOR localization similar to starvation state [Fig 5D,F]. Most importantly, in constitutively active dRagA expressing fat body cells, which normally have activated TOR localization, reducing Arf79F causes TOR to localize in a subcellular location similar to nutrient limitations [Fig 5E,F]. Thus, Arf79F needs to be investigated in further context of how it mediates TOR trafficking.

V. Conclusion

Our forward genetic approach though not fully successful at this time point, has uncovered additional positive players in the TOR pathway, the characterization of which in the future can further our understanding of TOR-mediated growth. Adopting a reverse candidate approach, in collaboration with K.L. Guan's group, we have identified two important positive regulators of TORC1 activity, dRag complex and Arf79F. dRags play an important role in amino acid sensing, one of the most elusive functions thus far of the TOR pathway. They act upstream or in parallel with Rheb to promote TORC1 mediated cell growth and mass accumulation.

One possible manner by which dRags can activate TOR is by mediating its subcellular localization to a compartment where TOR can subsequently get activated by coming in proximity to other activators, complex members or effectors. Immunohistochemistry with a FLAG antibody to visualize FLAG-TOR expression in fat body cells, indeed, demonstrates that activating signals (nutrient sufficiency) localize FLAG-TOR to cytoplasm and at cell membrane whereas inactivating signals (starvation) localize FLAG-TOR to the nucleus. This work has identified and characterized in a preliminary nature another novel positive regulator of TORC1, Arf79F, the *Drosophila* homolog of Arf1 for its role in mediating this TOR localization. We find that Arf79F is required for dRagA-mediated activation of TORC1 seen in fat body cells on amino acid starvation. The exact mechanics of Arf79F-mediated TOR translocation remain to be analyzed.

Identification of these positive regulators has answered long-standing questions in the field, and has contributed, in part, to our knowledge of the intricacies of the master regulatory TOR pathway that mediates nutrient stimulated growth and proliferation. They have now added on to the complexity of TOR network and it remains to be seen how Rag GTPases and Arf GTPases function relative to some of the other signals that impinge on TOR to achieve both spatial and temporal growth. Our study has thus opened up new avenues in TOR signaling that now are available for further investigation.

Bibliography

Aladzsity, I., M.L. Toth, T. Sigmond, E. Szabo, B. Bicsak, J. Barna, A. Regos, L. Orosz, A.L. Kovacs, and T. Vellai. 2007. Autophagy genes unc-51 and bec-1 are required for normal cell size in Caenorhabditis elegans. *Genetics*. 177:655-660.

Aronova, S., K. Wedaman, P.A. Aronov, K. Fontes, K. Ramos, B.D. Hammock, and T. Powers. 2008. Regulation of ceramide biosynthesis by TOR complex 2. *Cell.Metab*. 7:148-158.

Arsham, A.M., J.J. Howell, and M.C. Simon. 2003. A novel hypoxia-inducible factor-independent hypoxic response regulating mammalian target of rapamycin and its targets. *J.Biol.Chem*. 278:29655-29660.

Ashe, M.P., S.K. De Long, and A.B. Sachs. 2000. Glucose depletion rapidly inhibits translation initiation in yeast. *Mol.Biol.Cell*. 11:833-848.

Avruch, J., X. Long, S. Ortiz-Vega, J. Rapley, A. Papageorgiou, and N. Dai. 2008. Amino Acid Regulation of TOR Complex 1. *Am.J.Physiol.Endocrinol.Metab*.

Bachmann, R.A., J.H. Kim, A.L. Wu, I.H. Park, and J. Chen. 2006. A nuclear transport signal in mammalian target of rapamycin is critical for its cytoplasmic signaling to S6 kinase 1. *J.Biol.Chem*. 281:7357-7363.

Baehrecke, E.H. 2005. Autophagy: dual roles in life and death? *Nat.Rev.Mol.Cell Biol*. 6:505-510.

Bai, X., D. Ma, A. Liu, X. Shen, Q.J. Wang, Y. Liu, and Y. Jiang. 2007. Rheb activates mTOR by antagonizing its endogenous inhibitor, FKBP38. *Science*. 318:977-980.

Ballif, B.A., P.P. Roux, S.A. Gerber, J.P. MacKeigan, J. Blenis, and S.P. Gygi. 2005. Quantitative phosphorylation profiling of the ERK/p90 ribosomal S6 kinase-signaling cassette and its targets, the tuberous sclerosis tumor suppressors. *Proc.Natl.Acad.Sci.U.S.A*. 102:667-672.

Bateman, J.M., and H. McNeill. 2004. Temporal control of differentiation by the insulin receptor/tor pathway in Drosophila. *Cell*. 119:87-96.

Beck, T., A. Schmidt, and M.N. Hall. 1999. Starvation induces vacuolar targeting and degradation of the tryptophan permease in yeast. *J.Cell Biol*. 146:1227-1238.

Bell, A., L. Grunder, and A. Sorisky. 2000. Rapamycin inhibits human adipocyte differentiation in primary culture. *Obes.Res*. 8:249-254.

Berger, Z., B. Ravikumar, F.M. Menzies, L.G. Oroz, B.R. Underwood, M.N. Pangalos, I. Schmitt, U. Wullner, B.O. Evert, C.J. O'Kane, and D.C. Rubinsztein. 2006. Rapamycin alleviates toxicity of different aggregate-prone proteins. *Hum.Mol.Genet*. 15:433-442.

Bhaskar, P.T., and N. Hay. 2007. The two TORCs and Akt. *Dev.Cell*. 12:487-502.

Boll, M., M. Foltz, I. Rubio-Aliaga, and H. Daniel. 2003. A cluster of proton/amino acid transporter genes in the human and mouse genomes. *Genomics*. 82:47-56.

Britton, J.S., and B.A. Edgar. 1998. Environmental control of the cell cycle in Drosophila: nutrition activates mitotic and endoreplicative cells by distinct mechanisms. *Development*. 125:2149-2158.

Brugarolas, J.B., F. Vazquez, A. Reddy, W.R. Sellers, and W.G. Kaelin Jr. 2003. TSC2 regulates VEGF through mTOR-dependent and -independent pathways. *Cancer.Cell*. 4:147-158.

Brugarolas, J., K. Lei, R.L. Hurley, B.D. Manning, J.H. Reiling, E. Hafen, L.A. Witters, L.W. Ellisen, and W.G. Kaelin Jr. 2004. Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. *Genes Dev*. 18:2893-2904.

Buerger, C., B. DeVries, and V. Stambolic. 2006. Localization of Rheb to the endomembrane is critical for its signaling function. *Biochem.Biophys.Res.Commun*. 344:869-880.

Bun-Ya, M., S. Harashima, and Y. Oshima. 1992. Putative GTP-binding protein, Gtr1, associated with the function of the Pho84 inorganic phosphate transporter in Saccharomyces cerevisiae. *Mol.Cell.Biol*. 12:2958-2966.

Cai, S.L., A.R. Tee, J.D. Short, J.M. Bergeron, J. Kim, J. Shen, R. Guo, C.L. Johnson, K. Kiguchi, and C.L. Walker. 2006. Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning. *J.Cell Biol*. 173:279-289.

Carracedo, A., and P.P. Pandolfi. 2008. The PTEN-PI3K pathway: of feedbacks and cross-talks. *Oncogene*. 27:5527-5541.

Carroll, M., J. Dyer, and W.S. Sossin. 2006. Serotonin increases phosphorylation of synaptic 4EBP through TOR, but eukaryotic initiation factor 4E levels do not limit somatic cap-dependent translation in aplysia neurons. *Mol.Cell.Biol*. 26:8586-8598.

Chiang, G.G., and R.T. Abraham. 2005. Phosphorylation of mammalian target of rapamycin (mTOR) at Ser-2448 is mediated by p70S6 kinase. *J.Biol.Chem*. 280:25485-25490.

Choi, J.H., N.R. Adames, T.F. Chan, C. Zeng, J.A. Cooper, and X.F. Zheng. 2000. TOR signaling regulates microtubule structure and function. *Curr.Biol*. 10:861-864.

Choi, J.H., P.G. Bertram, R. Drenan, J. Carvalho, H.H. Zhou, and X.F. Zheng. 2002. The FKBP12-rapamycin-associated protein (FRAP) is a CLIP-170 kinase. *EMBO Rep*. 3:988-994.

Choi, Y.J., A. Di Nardo, I. Kramvis, L. Meikle, D.J. Kwiatkowski, M. Sahin, and X. He. 2008. Tuberous sclerosis complex proteins control axon formation. *Genes Dev*. 22:2485-2495.

Chung, J., C.J. Kuo, G.R. Crabtree, and J. Blenis. 1992. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. *Cell*. 69:1227-1236.

Cockcroft, S., G.M. Thomas, A. Fensome, B. Geny, E. Cunningham, I. Gout, I. Hiles, N.F. Totty, O. Truong, and J.J. Hsuan. 1994. Phospholipase D: a downstream effector of ARF in granulocytes. *Science*. 263:523-526.

Colombani, J., S. Raisin, S. Pantalacci, T. Radimerski, J. Montagne, and P. Leopold. 2003. A nutrient sensor mechanism controls Drosophila growth. *Cell*. 114:739-749.

Cyert, M.S. 2001. Genetic analysis of calmodulin and its targets in Saccharomyces cerevisiae. *Annu.Rev.Genet*. 35:647-672.

Dames, S.A., J.M. Mulet, K. Rathgeb-Szabo, M.N. Hall, and S. Grzesiek. 2005. The solution structure of the FATC domain of the protein kinase target of rapamycin suggests a role for redox-dependent structural and cellular stability. *J.Biol.Chem*. 280:20558-20564.

DeYoung, M.P., P. Horak, A. Sofer, D. Sgroi, and L.W. Ellisen. 2008. Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. *Genes Dev*. 22:239-251.

Dong, J., and D. Pan. 2004. Tsc2 is not a critical target of Akt during normal Drosophila development. *Genes Dev*. 18:2479-2484.

Drenan, R.M., X. Liu, P.G. Bertram, and X.F. Zheng. 2004. FKBP12-rapamycin-associated protein or mammalian target of rapamycin (FRAP/mTOR) localization in the endoplasmic reticulum and the Golgi apparatus. *J.Biol.Chem*. 279:772-778.

D'Souza-Schorey, C., and P. Chavrier. 2006. ARF proteins: roles in membrane traffic and beyond. *Nat.Rev.Mol.Cell Biol*. 7:347-358.

Dubouloz, F., O. Deloche, V. Wanke, E. Cameroni, and C. De Virgilio. 2005. The TOR and EGO protein complexes orchestrate microautophagy in yeast. *Mol.Cell*. 19:15-26.

Edinger, A.L., and C.B. Thompson. 2002. Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake. *Mol.Biol.Cell*. 13:2276-2288.

Fadri, M., A. Daquinag, S. Wang, T. Xue, and J. Kunz. 2005. The pleckstrin homology domain proteins Slm1 and Slm2 are required for actin cytoskeleton organization in yeast and bind phosphatidylinositol-4,5-bisphosphate and TORC2. *Mol.Biol.Cell*. 16:1883-1900.

Fang, Y., M. Vilella-Bach, R. Bachmann, A. Flanigan, and J. Chen. 2001. Phosphatidic acid-mediated mitogenic activation of mTOR signaling. *Science*. 294:1942-1945.

Fang, Y., I.H. Park, A.L. Wu, G. Du, P. Huang, M.A. Frohman, S.J. Walker, H.A. Brown, and J. Chen. 2003. PLD1 regulates mTOR signaling and mediates Cdc42 activation of S6K1. *Curr.Biol*. 13:2037-2044.

Floto, R.A., S. Sarkar, E.O. Perlstein, B. Kampmann, S.L. Schreiber, and D.C. Rubinsztein. 2007. Small molecule enhancers of rapamycin-induced TOR inhibition promote autophagy, reduce toxicity in Huntington's disease models and enhance killing of mycobacteria by macrophages. *Autophagy*. 3:620-622.

Foster, D.A., and L. Xu. 2003. Phospholipase D in cell proliferation and cancer. *Mol.Cancer.Res*. 1:789-800.

Friant, S., R. Lombardi, T. Schmelzle, M.N. Hall, and H. Riezman. 2001. Sphingoid base signaling via Pkh kinases is required for endocytosis in yeast. *EMBO J*. 20:6783-6792.

Frodin, M., T.L. Antal, B.A. Dummler, C.J. Jensen, M. Deak, S. Gammeltoft, and R.M. Biondi. 2002. A phosphoserine/threonine-binding pocket in AGC kinases and PDK1 mediates activation by hydrophobic motif phosphorylation. *EMBO J*. 21:5396-5407.

Furuya, N., J. Yu, M. Byfield, S. Pattingre, and B. Levine. 2005. The evolutionarily conserved domain of Beclin 1 is required for Vps34 binding, autophagy and tumor suppressor function. *Autophagy*. 1:46-52.

Gachet, Y., S. Tournier, M. Lee, A. Lazaris-Karatzas, T. Poulton, and U.A. Bommer. 1999. The growth-related, translationally controlled protein P23 has properties of a tubulin binding protein and associates transiently with microtubules during the cell cycle. *J.Cell.Sci.* 112 (Pt 8):1257-1271.

Gao, M., and C.A. Kaiser. 2006. A conserved GTPase-containing complex is required for intracellular sorting of the general amino-acid permease in yeast. *Nat.Cell Biol.* 8:657-667.

Gao, X., and D. Pan. 2001. TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. *Genes Dev.* 15:1383-1392.

Gao, X., Y. Zhang, P. Arrazola, O. Hino, T. Kobayashi, R.S. Yeung, B. Ru, and D. Pan. 2002. Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. *Nat.Cell Biol.* 4:699-704.

Gillingham, A.K., and S. Munro. 2007. The small G proteins of the Arf family and their regulators. *Annu.Rev.Cell Dev.Biol.* 23:579-611.

Goberdhan, D.C., D. Meredith, C.A. Boyd, and C. Wilson. 2005. PAT-related amino acid transporters regulate growth via a novel mechanism that does not require bulk transport of amino acids. *Development.* 132:2365-2375.

Gommel, D.U., A.R. Memon, A. Heiss, F. Lottspeich, J. Pfannstiel, J. Lechner, C. Reinhard, J.B. Helms, W. Nickel, and F.T. Wieland. 2001. Recruitment to Golgi membranes of ADP-ribosylation factor 1 is mediated by the cytoplasmic domain of p23. *EMBO J.* 20:6751-6760.

Greenspan RJ. 2004. Fly pushing – the theory and practice of Drosophila genetics

Grewal, S.S., J.R. Evans, and B.A. Edgar. 2007. Drosophila TIF-IA is required for ribosome synthesis and cell growth and is regulated by the TOR pathway. *J.Cell Biol.* 179:1105-1113.

Gstaiger, M., B. Luke, D. Hess, E.J. Oakeley, C. Wirbelauer, M. Blondel, M. Vigneron, M. Peter, and W. Krek. 2003. Control of nutrient-sensitive transcription programs by the unconventional prefoldin URI. *Science.* 302:1208-1212.

Gulati, P., L.D. Gaspers, S.G. Dann, M. Joaquin, T. Nobukuni, F. Natt, S.C. Kozma, A.P. Thomas, and G. Thomas. 2008. Amino acids activate mTOR complex 1 via Ca2+/CaM signaling to hVps34. *Cell.Metab.* 7:456-465.

Habib, S.L., D. Michel, E. Masliah, B. Thomas, H.S. Ko, T.M. Dawson, H. Abboud, R.A. Clark, and S.Z. Imam. 2008. Role of tuberin in neuronal degeneration. *Neurochem.Res.* 33:1113-1116.

Hall, D.J., S.S. Grewal, A.F. de la Cruz, and B.A. Edgar. 2007. Rheb-TOR signaling promotes protein synthesis, but not glucose or amino acid import, in Drosophila. *BMC Biol.* 5:10.

Hannan, K.M., Y. Brandenburger, A. Jenkins, K. Sharkey, A. Cavanaugh, L. Rothblum, T. Moss, G. Poortinga, G.A. McArthur, R.B. Pearson, and R.D. Hannan. 2003. mTOR-dependent regulation of ribosomal gene transcription requires S6K1 and is mediated by phosphorylation of the carboxy-terminal activation domain of the nucleolar transcription factor UBF. *Mol.Cell.Biol.* 23:8862-8877.

Hara, K., K. Yonezawa, Q.P. Weng, M.T. Kozlowski, C. Belham, and J. Avruch. 1998. Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J.Biol.Chem.* 273:14484-14494.

Harrington, L.S., G.M. Findlay, A. Gray, T. Tolkacheva, S. Wigfield, H. Rebholz, J. Barnett, N.R. Leslie, S. Cheng, P.R. Shepherd, I. Gout, C.P. Downes, and R.F. Lamb. 2004. The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. *J.Cell Biol*. 166:213-223.

Heitman, J., N.R. Movva, and M.N. Hall. 1991. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science*. 253:905-909.

Helliwell, S.B., A. Schmidt, Y. Ohya, and M.N. Hall. 1998. The Rho1 effector Pkc1, but not Bni1, mediates signalling from Tor2 to the actin cytoskeleton. *Curr.Biol*. 8:1211-1214.

Hennig, K.M., and T.P. Neufeld. 2002. Inhibition of cellular growth and proliferation by dTOR overexpression in Drosophila. *Genesis*. 34:107-110.

Hennig, K.M., J. Colombani, and T.P. Neufeld. 2006. TOR coordinates bulk and targeted endocytosis in the Drosophila melanogaster fat body to regulate cell growth. *J.Cell Biol*. 173:963-974.

Heuvingh, J., M. Franco, P. Chavrier, and C. Sykes. 2007. ARF1-mediated actin polymerization produces movement of artificial vesicles. *Proc.Natl.Acad.Sci.U.S.A*. 104:16928-16933.

Hillier, L.W., G.T. Marth, A.R. Quinlan, D. Dooling, G. Fewell, D. Barnett, P. Fox, J.I. Glasscock, M. Hickenbotham, W. Huang, V.J. Magrini, R.J. Richt, S.N. Sander, D.A. Stewart, M. Stromberg, E.F. Tsung, T. Wylie, T. Schedl, R.K. Wilson, and E.R. Mardis. 2008. Whole-genome sequencing and variant discovery in C. elegans. *Nat.Methods*. 5:183-188.

Hirose, E., N. Nakashima, T. Sekiguchi, and T. Nishimoto. 1998. RagA is a functional homologue of S. cerevisiae Gtr1p involved in the Ran/Gsp1-GTPase pathway. *J.Cell.Sci*. 111 (Pt 1):11-21.

Hoeller, O., and R.R. Kay. 2007. Chemotaxis in the absence of PIP3 gradients. *Curr.Biol*. 17:813-817.

Holliday, R. 1989. Food, reproduction and longevity: is the extended lifespan of calorie-restricted animals an evolutionary adaptation? *Bioessays*. 10:125-127.

Holz, M.K., B.A. Ballif, S.P. Gygi, and J. Blenis. 2005. mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell*. 123:569-580.

Holz, M.K., and J. Blenis. 2005. Identification of S6 kinase 1 as a novel mammalian target of rapamycin (mTOR)-phosphorylating kinase. *J.Biol.Chem*. 280:26089-26093.

Homyk, T., D.A. Sinclair, D.T. Wong, and T.A. Grigliatti. 1986. Recovery and Characterization of Temperature-Sensitive Mutations Affecting Adult Viability in DROSOPHILA MELANOGASTER. *Genetics*. 113:367-389.

Hsu, Y.C., J.J. Chern, Y. Cai, M. Liu, and K.W. Choi. 2007. Drosophila TCTP is essential for growth and proliferation through regulation of dRheb GTPase. *Nature*. 445:785-788.

Hudson, C.C., M. Liu, G.G. Chiang, D.M. Otterness, D.C. Loomis, F. Kaper, A.J. Giaccia, and R.T. Abraham. 2002. Regulation of hypoxia-inducible factor 1alpha expression and function by the mammalian target of rapamycin. *Mol.Cell.Biol*. 22:7004-7014.

Hwang, M., C.A. Perez, L. Moretti, and B. Lu. 2008. The mTOR signaling network: insights from its role during embryonic development. *Curr.Med.Chem*. 15:1192-1208.

Hwangbo, D.S., B. Gershman, M.P. Tu, M. Palmer, and M. Tatar. 2004. Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. *Nature*. 429:562-566.

Iiboshi, Y., P.J. Papst, H. Kawasome, H. Hosoi, R.T. Abraham, P.J. Houghton, and N. Terada. 1999. Amino acid-dependent control of p70(s6k). Involvement of tRNA aminoacylation in the regulation. *J.Biol.Chem*. 274:1092-1099.

Ikenoue, T., K. Inoki, Q. Yang, X. Zhou, and K.L. Guan. 2008. Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. *EMBO J*. 27:1919-1931.

Inoki, K., Y. Li, T. Zhu, J. Wu, and K.L. Guan. 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat.Cell Biol*. 4:648-657.

Inoki, K., T. Zhu, and K.L. Guan. 2003. TSC2 mediates cellular energy response to control cell growth and survival. *Cell*. 115:577-590.

Inoki, K., H. Ouyang, T. Zhu, C. Lindvall, Y. Wang, X. Zhang, Q. Yang, C. Bennett, Y. Harada, K. Stankunas, C.Y. Wang, X. He, O.A. MacDougald, M. You, B.O. Williams, and K.L. Guan. 2006. TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. *Cell*. 126:955-968.

Itzhaki, R.F., S.L. Cosby, and M.A. Wozniak. 2008. Herpes simplex virus type 1 and Alzheimer's disease: the autophagy connection. *J.Neurovirol*. 14:1-4.

Jacinto, E., and M.N. Hall. 2003. Tor signalling in bugs, brain and brawn. *Nat.Rev.Mol.Cell Biol*. 4:117-126.

Jacinto, E., R. Loewith, A. Schmidt, S. Lin, M.A. Ruegg, A. Hall, and M.N. Hall. 2004. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat.Cell Biol*. 6:1122-1128.

Jacinto, E., and A. Lorberg. 2008. TOR regulation of AGC kinases in yeast and mammals. *Biochem.J*. 410:19-37.

Jia, K., D. Chen, and D.L. Riddle. 2004. The TOR pathway interacts with the insulin signaling pathway to regulate C. elegans larval development, metabolism and life span. *Development*. 131:3897-3906.

Juhasz, G., J.H. Hill, Y. Yan, M. Sass, E.H. Baehrecke, J.M. Backer, and T.P. Neufeld. 2008. The class III PI(3)K Vps34 promotes autophagy and endocytosis but not TOR signaling in Drosophila. *J.Cell Biol*. 181:655-666.

Kabeya, Y., N. Mizushima, T. Ueno, A. Yamamoto, T. Kirisako, T. Noda, E. Kominami, Y. Ohsumi, and T. Yoshimori. 2000. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J*. 19:5720-5728.

Kabeya, Y., N. Mizushima, A. Yamamoto, S. Oshitani-Okamoto, Y. Ohsumi, and T. Yoshimori. 2004. LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. *J.Cell.Sci*. 117:2805-2812.

Kahn, R.A., and A.G. Gilman. 1986. The protein cofactor necessary for ADP-ribosylation of Gs by cholera toxin is itself a GTP binding protein. *J.Biol.Chem*. 261:7906-7911.

Kamada, Y., Y. Fujioka, N.N. Suzuki, F. Inagaki, S. Wullschleger, R. Loewith, M.N. Hall, and Y. Ohsumi. 2005. Tor2 directly phosphorylates the AGC kinase Ypk2 to regulate actin polarization. *Mol.Cell.Biol*. 25:7239-7248.

Kamimura, Y., Y. Xiong, P.A. Iglesias, O. Hoeller, P. Bolourani, and P.N. Devreotes. 2008. PIP3-independent activation of TorC2 and PKB at the cell's leading edge mediates chemotaxis. *Curr.Biol*. 18:1034-1043.

Kapahi, P., and B. Zid. 2004. TOR pathway: linking nutrient sensing to life span. *Sci.Aging Knowledge Environ*. 2004:PE34.

Kapahi, P., B.M. Zid, T. Harper, D. Koslover, V. Sapin, and S. Benzer. 2004. Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. *Curr.Biol*. 14:885-890.

Kim, D.H., D.D. Sarbassov, S.M. Ali, J.E. King, R.R. Latek, H. Erdjument-Bromage, P. Tempst, and D.M. Sabatini. 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell*. 110:163-175.

Kim, D.H., D.D. Sarbassov, S.M. Ali, R.R. Latek, K.V. Guntur, H. Erdjument-Bromage, P. Tempst, and D.M. Sabatini. 2003. GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. *Mol.Cell*. 11:895-904.

Kim, E., P. Goraksha-Hicks, L. Li, T.P. Neufeld, and K.L. Guan. 2008. Regulation of TORC1 by Rag GTPases in nutrient response. *Nat.Cell Biol*. 10:935-945.

Kim, J.E., and J. Chen. 2004. Regulation of Peroxisome Proliferator-Activated Receptor-Gamma Activity by Mammalian Target of Rapamycin and Amino Acids in Adipogenesis. *Diabetes*. 53:2748-2756.

Kuma, A., M. Hatano, M. Matsui, A. Yamamoto, H. Nakaya, T. Yoshimori, Y. Ohsumi, T. Tokuhisa, and N. Mizushima. 2004. The role of autophagy during the early neonatal starvation period. *Nature*. 432:1032-1036.

Kunz, J., U. Schneider, I. Howald, A. Schmidt, and M.N. Hall. 2000. HEAT repeats mediate plasma membrane localization of Tor2p in yeast. *J.Biol.Chem*. 275:37011-37020.

Kwiatkowski, D.J., and B.D. Manning. 2005. Tuberous sclerosis: a GAP at the crossroads of multiple signaling pathways. *Hum.Mol.Genet*. 14 Spec No. 2:R251-8.

Lamouille, S., and R. Derynck. 2007. Cell size and invasion in TGF-beta-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. *J.Cell Biol*. 178:437-451.

Lee, F.J., L.A. Stevens, L.M. Hall, J.J. Murtagh Jr, Y.L. Kao, J. Moss, and M. Vaughan. 1994. Characterization of class II and class III ADP-ribosylation factor genes and proteins in Drosophila melanogaster. *J.Biol.Chem*. 269:21555-21560.

Lee, S., F.I. Comer, A. Sasaki, I.X. McLeod, Y. Duong, K. Okumura, J.R. Yates 3rd, C.A. Parent, and R.A. Firtel. 2005. TOR complex 2 integrates cell movement during chemotaxis and signal relay in Dictyostelium. *Mol.Biol.Cell*. 16:4572-4583.

Lee, S.J., R. Feldman, and P.H. O'Farrell. 2008. An RNA interference screen identifies a novel regulator of target of rapamycin that mediates hypoxia suppression of translation in Drosophila S2 cells. *Mol.Biol.Cell.* 19:4051-4061.

Leevers, S.J., and H. McNeill. 2005. Controlling the size of organs and organisms. *Curr.Opin.Cell Biol.* 17:604-609.

Li, H., C.K. Tsang, M. Watkins, P.G. Bertram, and X.F. Zheng. 2006. Nutrient regulates Tor1 nuclear localization and association with rDNA promoter. *Nature.* 442:1058-1061.

Li, Y., W.G. Kelly, J.M. Logsdon Jr, A.M. Schurko, B.D. Harfe, K.L. Hill-Harfe, and R.A. Kahn. 2004. Functional genomic analysis of the ADP-ribosylation factor family of GTPases: phylogeny among diverse eukaryotes and function in C. elegans. *FASEB J.* 18:1834-1850.

Lizcano, J.M., S. Alrubaie, A. Kieloch, M. Deak, S.J. Leevers, and D.R. Alessi. 2003. Insulin-induced Drosophila S6 kinase activation requires phosphoinositide 3-kinase and protein kinase B. *Biochem.J.* 374:297-306.

Llovet, J.M., and J. Bruix. 2008. Molecular targeted therapies in hepatocellular carcinoma. *Hepatology.* 48:1312-1327.

Loewith, R., E. Jacinto, S. Wullschleger, A. Lorberg, J.L. Crespo, D. Bonenfant, W. Oppliger, P. Jenoe, and M.N. Hall. 2002. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol.Cell.* 10:457-468.

Long, X., C. Spycher, Z.S. Han, A.M. Rose, F. Muller, and J. Avruch. 2002. TOR deficiency in C. elegans causes developmental arrest and intestinal atrophy by inhibition of mRNA translation. *Curr.Biol.* 12:1448-1461.

Long, X., Y. Lin, S. Ortiz-Vega, K. Yonezawa, and J. Avruch. 2005. Rheb binds and regulates the mTOR kinase. *Curr.Biol.* 15:702-713.

Long, X., S. Ortiz-Vega, Y. Lin, and J. Avruch. 2005. Rheb binding to mammalian target of rapamycin (mTOR) is regulated by amino acid sufficiency. *J.Biol.Chem.* 280:23433-23436.

Long, X., Y. Lin, S. Ortiz-Vega, S. Busch, and J. Avruch. 2007. The Rheb switch 2 segment is critical for signaling to target of rapamycin complex 1. *J.Biol.Chem.* 282:18542-18551.

Lundmark, R., G.J. Doherty, Y. Vallis, B.J. Peter, and H.T. McMahon. 2008. Arf family GTP loading is activated by, and generates, positive membrane curvature. *Biochem.J.* 414:189-194.

Ma, D., X. Bai, S. Guo, and Y. Jiang. 2008. The switch I region of Rheb is critical for its interaction with FKBP38. *J.Biol.Chem.* 283:25963-25970.

Ma, J., R. Jin, C.J. Dobry, S.K. Lawson, and A. Kumar. 2007. Overexpression of autophagy-related genes inhibits yeast filamentous growth. *Autophagy.* 3:604-609.

Ma, L., Z. Chen, H. Erdjument-Bromage, P. Tempst, and P.P. Pandolfi. 2005. Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. *Cell.* 121:179-193.

Makky, K., J. Tekiela, and A.N. Mayer. 2007. Target of rapamycin (TOR) signaling controls epithelial morphogenesis in the vertebrate intestine. *Dev.Biol*. 303:501-513.

Martin, J.F., E. Hersperger, A. Simcox, and A. Shearn. 2000. Minidiscs Encodes a Putative Amino Acid Transporter Subunit Required Non-Autonomously for Imaginal Cell Proliferation. *Mech.Dev*. 92:155-167.

Mayer, C., J. Zhao, X. Yuan, and I. Grummt. 2004. mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. *Genes Dev*. 18:423-434.

McCormack, J.G., and R.M. Denton. 1993. Mitochondrial Ca2+ transport and the role of intramitochondrial Ca2+ in the regulation of energy metabolism. *Dev.Neurosci*. 15:165-173.

McNeill, H., G.M. Craig, and J.M. Bateman. 2008. Regulation of neurogenesis and epidermal growth factor receptor signaling by the insulin receptor/target of rapamycin pathway in Drosophila. *Genetics*. 179:843-853.

Meikle, L., K. Pollizzi, A. Egnor, I. Kramvis, H. Lane, M. Sahin, and D.J. Kwiatkowski. 2008. Response of a neuronal model of tuberous sclerosis to mammalian target of rapamycin (mTOR) inhibitors: effects on mTORC1 and Akt signaling lead to improved survival and function. *J.Neurosci*. 28:5422-5432.

Meikle, L., D.M. Talos, H. Onda, K. Pollizzi, A. Rotenberg, M. Sahin, F.E. Jensen, and D.J. Kwiatkowski. 2007. A mouse model of tuberous sclerosis: neuronal loss of Tsc1 causes dysplastic and ectopic neurons, reduced myelination, seizure activity, and limited survival. *J.Neurosci*. 27:5546-5558.

Melendez, A., and T.P. Neufeld. 2008. The cell biology of autophagy in metazoans: a developing story. *Development*. 135:2347-2360.

Menand, B., T. Desnos, L. Nussaume, F. Berger, D. Bouchez, C. Meyer, and C. Robaglia. 2002. Expression and disruption of the Arabidopsis TOR (target of rapamycin) gene. *Proc.Natl.Acad.Sci.U.S.A*. 99:6422-6427.

Mulet, J.M., D.E. Martin, R. Loewith, and M.N. Hall. 2006. Mutual antagonism of target of rapamycin and calcineurin signaling. *J.Biol.Chem*. 281:33000-33007.

Murtagh, J.J.,Jr, M.R. Mowatt, C.M. Lee, F.J. Lee, K. Mishima, T.E. Nash, J. Moss, and M. Vaughan. 1992. Guanine nucleotide-binding proteins in the intestinal parasite Giardia lamblia. Isolation of a gene encoding an approximately 20-kDa ADP-ribosylation factor. *J.Biol.Chem*. 267:9654-9662.

Nakashima, N., E. Noguchi, and T. Nishimoto. 1999. Saccharomyces cerevisiae putative G protein, Gtr1p, which forms complexes with itself and a novel protein designated as Gtr2p, negatively regulates the Ran/Gsp1p G protein cycle through Gtr2p. *Genetics*. 152:853-867.

Narayanan, U., V. Nalavadi, M. Nakamoto, G. Thomas, S. Ceman, G.J. Bassell, and S.T. Warren. 2008. S6K1 phosphorylates and regulates fragile X mental retardation protein (FMRP) with the neuronal protein synthesis-dependent mammalian target of rapamycin (mTOR) signaling cascade. *J.Biol.Chem*. 283:18478-18482.

Nelsen, C.J., D.G. Rickheim, M.M. Tucker, L.K. Hansen, and J.H. Albrecht. 2003. Evidence that cyclin D1 mediates both growth and proliferation downstream of TOR in hepatocytes. *J.Biol.Chem*. 278:3656-3663.

Neufeld, T.P. 2003. Body building: regulation of shape and size by PI3K/TOR signaling during development. *Mech.Dev*. 120:1283-1296.

Nobukuni, T., M. Joaquin, M. Roccio, S.G. Dann, S.Y. Kim, P. Gulati, M.P. Byfield, J.M. Backer, F. Natt, J.L. Bos, F.J. Zwartkruis, and G. Thomas. 2005. Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. *Proc.Natl.Acad.Sci.U.S.A*. 102:14238-14243.

Nojima, H., C. Tokunaga, S. Eguchi, N. Oshiro, S. Hidayat, K. Yoshino, K. Hara, N. Tanaka, J. Avruch, and K. Yonezawa. 2003. The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. *J.Biol.Chem*. 278:15461-15464.

Obeid, L.M., and Y.A. Hannun. 2003. Ceramide, stress, and a "LAG" in aging. *Sci.Aging Knowledge Environ*. 2003:PE27.

Oldham, S., J. Montagne, T. Radimerski, G. Thomas, and E. Hafen. 2000. Genetic and biochemical characterization of dTOR, the Drosophila homolog of the target of rapamycin. *Genes Dev*. 14:2689-2694.

Onodera, J., and Y. Ohsumi. 2005. Autophagy is required for maintenance of amino acid levels and protein synthesis under nitrogen starvation. *J.Biol.Chem*. 280:31582-31586.

Orvedahl, A., and B. Levine. 2008. Eating the enemy within: autophagy in infectious diseases. *Cell Death Differ*.

Parks, A.L., K.R. Cook, M. Belvin, N.A. Dompe, R. Fawcett, K. Huppert, L.R. Tan, C.G. Winter, K.P. Bogart, J.E. Deal, M.E. Deal-Herr, D. Grant, M. Marcinko, W.Y. Miyazaki, S. Robertson, K.J. Shaw, M. Tabios, V. Vysotskaia, L. Zhao, R.S. Andrade, K.A. Edgar, E. Howie, K. Killpack, B. Milash, A. Norton, D. Thao, K. Whittaker, M.A. Winner, L. Friedman, J. Margolis, M.A. Singer, C. Kopczynski, D. Curtis, T.C. Kaufman, G.D. Plowman, G. Duyk, and H.L. Francis-Lang. 2004. Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome. *Nat.Genet*. 36:288-292.

Patel, P.H., N. Thapar, L. Guo, M. Martinez, J. Maris, C.L. Gau, J.A. Lengyel, and F. Tamanoi. 2003. Drosophila Rheb GTPase is required for cell cycle progression and cell growth. *J.Cell.Sci*. 116:3601-3610.

Pedruzzi, I., F. Dubouloz, E. Cameroni, V. Wanke, J. Roosen, J. Winderickx, and C. De Virgilio. 2003. TOR and PKA signaling pathways converge on the protein kinase Rim15 to control entry into G0. *Mol.Cell*. 12:1607-1613.

Pelkmans, L., E. Fava, H. Grabner, M. Hannus, B. Habermann, E. Krausz, and M. Zerial. 2005. Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. *Nature*. 436:78-86.

Pende, M., S.H. Um, V. Mieulet, M. Sticker, V.L. Goss, J. Mestan, M. Mueller, S. Fumagalli, S.C. Kozma, and G. Thomas. 2004. S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. *Mol.Cell.Biol*. 24:3112-3124.

Peng, T., T.R. Golub, and D.M. Sabatini. 2002. The immunosuppressant rapamycin mimics a starvation-like signal distinct from amino acid and glucose deprivation. *Mol.Cell.Biol*. 22:5575-5584.

Potter, C.J., H. Huang, and T. Xu. 2001. Drosophila Tsc1 functions with Tsc2 to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size. *Cell*. 105:357-368.

Potter, C.J., L.G. Pedraza, and T. Xu. 2002. Akt regulates growth by directly phosphorylating Tsc2. *Nat.Cell Biol*. 4:658-665.

Powers, T., and P. Walter. 1999. Regulation of ribosome biogenesis by the rapamycin-sensitive TOR-signaling pathway in Saccharomyces cerevisiae. *Mol.Biol.Cell*. 10:987-1000.

Powner, D.J., and M.J. Wakelam. 2002. The regulation of phospholipase D by inositol phospholipids and small GTPases. *FEBS Lett*. 531:62-64.

Puig, O., M.T. Marr, M.L. Ruhf, and R. Tjian. 2003. Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway. *Genes Dev*. 17:2006-2020.

Radimerski, T., J. Montagne, M. Hemmings-Mieszczak, and G. Thomas. 2002. Lethality of Drosophila lacking TSC tumor suppressor function rescued by reducing dS6K signaling. *Genes Dev*. 16:2627-2632.

Ravikumar, B., R. Duden, and D.C. Rubinsztein. 2002. Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. *Hum.Mol.Genet*. 11:1107-1117.

Reggiori, F., and D.J. Klionsky. 2005. Autophagosomes: biogenesis from scratch? *Curr.Opin.Cell Biol*. 17:415-422.

Rehmann, H., M. Bruning, C. Berghaus, M. Schwarten, K. Kohler, H. Stocker, R. Stoll, F.J. Zwartkruis, and A. Wittinghofer. 2008. Biochemical characterisation of TCTP questions its function as a guanine nucleotide exchange factor for Rheb. *FEBS Lett*. 582:3005-3010.

Reiling, J.H., and E. Hafen. 2004. The hypoxia-induced paralogs Scylla and Charybdis inhibit growth by down-regulating S6K activity upstream of TSC in Drosophila. *Genes Dev*. 18:2879-2892.

Rogers, G.W.,Jr, N.J. Richter, and W.C. Merrick. 1999. Biochemical and kinetic characterization of the RNA helicase activity of eukaryotic initiation factor 4A. *J.Biol.Chem*. 274:12236-12244.

Rohde, J.R., and M.E. Cardenas. 2003. The tor pathway regulates gene expression by linking nutrient sensing to histone acetylation. *Mol.Cell.Biol*. 23:629-635.

Rohde, J.R., R. Bastidas, R. Puria, and M.E. Cardenas. 2008. Nutritional control via Tor signaling in Saccharomyces cerevisiae. *Curr.Opin.Microbiol*. 11:153-160.

Rosner, M., M. Hanneder, N. Siegel, A. Valli, C. Fuchs, and M. Hengstschlager. 2008. The mTOR pathway and its role in human genetic diseases. *Mutat.Res*. 659:284-292.

Roux, P.P., B.A. Ballif, R. Anjum, S.P. Gygi, and J. Blenis. 2004. Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. *Proc.Natl.Acad.Sci.U.S.A*. 101:13489-13494.

Rusten, T.E., K. Lindmo, G. Juhasz, M. Sass, P.O. Seglen, A. Brech, and H. Stenmark. 2004. Programmed autophagy in the Drosophila fat body is induced by ecdysone through regulation of the PI3K pathway. *Dev.Cell*. 7:179-192.

Ruvinsky, I., N. Sharon, T. Lerer, H. Cohen, M. Stolovich-Rain, T. Nir, Y. Dor, P. Zisman, and O. Meyuhas. 2005. Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. *Genes Dev*. 19:2199-2211.

Sancak, Y., C.C. Thoreen, T.R. Peterson, R.A. Lindquist, S.A. Kang, E. Spooner, S.A. Carr, and D.M. Sabatini. 2007. PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. *Mol.Cell*. 25:903-915.

Sancak, Y., T.R. Peterson, Y.D. Shaul, R.A. Lindquist, C.C. Thoreen, L. Bar-Peled, and D.M. Sabatini. 2008. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science*. 320:1496-1501.

Sarbassov, D.D., S.M. Ali, D.H. Kim, D.A. Guertin, R.R. Latek, H. Erdjument-Bromage, P. Tempst, and D.M. Sabatini. 2004. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr.Biol*. 14:1296-1302.

Sarbassov, D.D., D.A. Guertin, S.M. Ali, and D.M. Sabatini. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science*. 307:1098-1101.

Sarbassov, D.D., S.M. Ali, S. Sengupta, J.H. Sheen, P.P. Hsu, A.F. Bagley, A.L. Markhard, and D.M. Sabatini. 2006. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol.Cell*. 22:159-168.

Sarkar, S., and D.C. Rubinsztein. 2008. Huntington's disease: degradation of mutant huntingtin by autophagy. *FEBS J*. 275:4263-4270.

Saucedo, L.J., X. Gao, D.A. Chiarelli, L. Li, D. Pan, and B.A. Edgar. 2003. Rheb promotes cell growth as a component of the insulin/TOR signalling network. *Nat.Cell Biol*. 5:566-571.

Schmelzle, T., T. Beck, D.E. Martin, and M.N. Hall. 2004. Activation of the RAS/cyclic AMP pathway suppresses a TOR deficiency in yeast. *Mol.Cell.Biol*. 24:338-351.

Schmidt, A., J. Kunz, and M.N. Hall. 1996. TOR2 is required for organization of the actin cytoskeleton in yeast. *Proc.Natl.Acad.Sci.U.S.A*. 93:13780-13785.

Schmidt, A., M. Bickle, T. Beck, and M.N. Hall. 1997. The yeast phosphatidylinositol kinase homolog TOR2 activates RHO1 and RHO2 via the exchange factor ROM2. *Cell*. 88:531-542.

Scott, R.C., O. Schuldiner, and T.P. Neufeld. 2004. Role and regulation of starvation-induced autophagy in the Drosophila fat body. *Dev.Cell*. 7:167-178.

Scott, R.C., G. Juhasz, and T.P. Neufeld. 2007. Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death. *Curr.Biol*. 17:1-11.

Sedding, D.G. 2008. FoxO transcription factors in oxidative stress response and ageing--a new fork on the way to longevity? *Biol.Chem*. 389:279-283.

Sekiguchi, T., Y. Todaka, Y. Wang, E. Hirose, N. Nakashima, and T. Nishimoto. 2004. A novel human nucleolar protein, Nop132, binds to the G proteins, RRAG A/C/D. *J.Biol.Chem*. 279:8343-8350.

Shah, O.J., Z. Wang, and T. Hunter. 2004. Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies. *Curr.Biol.* 14:1650-1656.

Shaw, R.J., M. Kosmatka, N. Bardeesy, R.L. Hurley, L.A. Witters, R.A. DePinho, and L.C. Cantley. 2004. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc.Natl.Acad.Sci.U.S.A.* 101:3329-3335.

Smith, E.M., S.G. Finn, A.R. Tee, G.J. Browne, and C.G. Proud. 2005. The tuberous sclerosis protein TSC2 is not required for the regulation of the mammalian target of rapamycin by amino acids and certain cellular stresses. *J.Biol.Chem.* 280:18717-18727.

Sofer, A., K. Lei, C.M. Johannessen, and L.W. Ellisen. 2005. Regulation of mTOR and cell growth in response to energy stress by REDD1. *Mol.Cell.Biol.* 25:5834-5845.

Song, J., L. Wu, Z. Chen, R.A. Kohanski, and L. Pick. 2003. Axons guided by insulin receptor in Drosophila visual system. *Science.* 300:502-505.

Song, Q., and L.I. Gilbert. 1994. S6 phosphorylation results from prothoracicotropic hormone stimulation of insect prothoracic glands: a role for S6 kinase. *Dev.Genet.* 15:332-338.

St Johnston, D. 2002. The art and design of genetic screens: Drosophila melanogaster. *Nat.Rev.Genet.* 3:176-188.

Stocker, H., T. Radimerski, B. Schindelholz, F. Wittwer, P. Belawat, P. Daram, S. Breuer, G. Thomas, and E. Hafen. 2003. Rheb is an essential regulator of S6K in controlling cell growth in Drosophila. *Nat.Cell Biol.* 5:559-565.

Sun, Y., Y. Fang, M.S. Yoon, C. Zhang, M. Roccio, F.J. Zwartkruis, M. Armstrong, H.A. Brown, and J. Chen. 2008. Phospholipase D1 is an effector of Rheb in the mTOR pathway. *Proc.Natl.Acad.Sci.U.S.A.* 105:8286-8291.

Tabuchi, M., A. Audhya, A.B. Parsons, C. Boone, and S.D. Emr. 2006. The phosphatidylinositol 4,5-biphosphate and TORC2 binding proteins Slm1 and Slm2 function in sphingolipid regulation. *Mol.Cell.Biol.* 26:5861-5875.

Takahashi, K., M. Nakagawa, S.G. Young, and S. Yamanaka. 2005. Differential membrane localization of ERas and Rheb, two Ras-related proteins involved in the phosphatidylinositol 3-kinase/mTOR pathway. *J.Biol.Chem.* 280:32768-32774.

Tapon, N., N. Ito, B.J. Dickson, J.E. Treisman, and I.K. Hariharan. 2001. The Drosophila tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. *Cell.* 105:345-355.

Teleman, A.A., Y.W. Chen, and S.M. Cohen. 2005. Drosophila Melted modulates FOXO and TOR activity. *Dev.Cell.* 9:271-281.

Um, S.H., F. Frigerio, M. Watanabe, F. Picard, M. Joaquin, M. Sticker, S. Fumagalli, P.R. Allegrini, S.C. Kozma, J. Auwerx, and G. Thomas. 2004. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature.* 431:200-205.

Vellai, T., K. Takacs-Vellai, Y. Zhang, A.L. Kovacs, L. Orosz, and F. Muller. 2003. Genetics: influence of TOR kinase on lifespan in C. elegans. *Nature.* 426:620.

Wang, L., T.E. Harris, R.A. Roth, and J.C. Lawrence Jr. 2007. PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding. *J.Biol.Chem.* 282:20036-20044.

Wang, L., T.E. Harris, and J.C. Lawrence Jr. 2008. Regulation of proline-rich Akt substrate of 40 kDa (PRAS40) function by mammalian target of rapamycin complex 1 (mTORC1)-mediated phosphorylation. *J.Biol.Chem.* 283:15619-15627.

Wang, X., W. Li, M. Williams, N. Terada, D.R. Alessi, and C.G. Proud. 2001. Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. *EMBO J.* 20:4370-4379.

Wang, X., B.D. Fonseca, H. Tang, R. Liu, A. Elia, M.J. Clemens, U.A. Bommer, and C.G. Proud. 2008. Re-evaluating the roles of proposed modulators of mammalian target of rapamycin complex 1 (mTORC1) signaling. *J.Biol.Chem.*

Wang, Y., N. Nakashima, T. Sekiguchi, and T. Nishimoto. 2005. Saccharomyces cerevisiae GTPase complex: Gtr1p-Gtr2p regulates cell-proliferation through Saccharomyces cerevisiae Ran-binding protein, Yrb2p. *Biochem.Biophys.Res.Commun.* 336:639-645.

Wedaman, K.P., A. Reinke, S. Anderson, J. Yates 3rd, J.M. McCaffery, and T. Powers. 2003. Tor kinases are in distinct membrane-associated protein complexes in Saccharomyces cerevisiae. *Mol.Biol.Cell.* 14:1204-1220.

Wilson, R.J., J.L. Goodman, V.B. Strelets, and FlyBase Consortium. 2008. FlyBase: integration and improvements to query tools. *Nucleic Acids Res.* 36:D588-93.

Withers, D.J., D.M. Ouwens, B.T. Nave, G.C. van der Zon, C.M. Alarcon, M.E. Cardenas, J. Heitman, J.A. Maassen, and P.R. Shepherd. 1997. Expression, enzyme activity, and subcellular localization of mammalian target of rapamycin in insulin-responsive cells. *Biochem.Biophys.Res.Commun.* 241:704-709.

Yang, Q., K. Inoki, E. Kim, and K.L. Guan. 2006. TSC1/TSC2 and Rheb have different effects on TORC1 and TORC2 activity. *Proc.Natl.Acad.Sci.U.S.A.* 103:6811-6816.

Yang, W., A.P. Tabancay Jr, J. Urano, and F. Tamanoi. 2001. Failure to farnesylate Rheb protein contributes to the enrichment of G0/G1 phase cells in the Schizosaccharomyces pombe farnesyltransferase mutant. *Mol.Microbiol.* 41:1339-1347.

Yue, Z., S. Jin, C. Yang, A.J. Levine, and N. Heintz. 2003. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc.Natl.Acad.Sci.U.S.A.* 100:15077-15082.

Zhai, R.G., P.R. Hiesinger, T.W. Koh, P. Verstreken, K.L. Schulze, Y. Cao, H. Jafar-Nejad, K.K. Norga, H. Pan, V. Bayat, M.P. Greenbaum, and H.J. Bellen. 2003. Mapping Drosophila mutations with molecularly defined P element insertions. *Proc.Natl.Acad.Sci.U.S.A.* 100:10860-10865.

Zhang, H., J.P. Stallock, J.C. Ng, C. Reinhard, and T.P. Neufeld. 2000. Regulation of cellular growth by the Drosophila target of rapamycin dTOR. *Genes Dev.* 14:2712-2724.

Zhang, Y., C.J. Billington Jr, D. Pan, and T.P. Neufeld. 2006. Drosophila target of rapamycin kinase functions as a multimer. *Genetics.* 172:355-362.

Zhang, Y., X. Gao, L.J. Saucedo, B. Ru, B.A. Edgar, and D. Pan. 2003. Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. *Nat.Cell Biol*. 5:578-581.

Zhang, Y., H. Qi, R. Taylor, W. Xu, L.F. Liu, and S. Jin. 2007. The role of autophagy in mitochondria maintenance: characterization of mitochondrial functions in autophagy-deficient S. cerevisiae strains. *Autophagy*. 3:337-346.

Zurita-Martinez, S.A., R. Puria, X. Pan, J.D. Boeke, and M.E. Cardenas. 2007. Efficient Tor signaling requires a functional class C Vps protein complex in Saccharomyces cerevisiae. *Genetics*. 176:2139-2150.

Appendix 1. Permission to use copyrighted material

Dear Dr. Goraksha-Hicks,

Thank you for letting me know. In that case, you will be pleased to know that permission is not needed for this use as the Licence to Publish explains.

Thank You for contacting the nature publishing group. As an author, you have the right to use this manuscript and figures, as per the licence-to-publish you signed:

Ownership of copyright in the article remains with the Authors, and provided that, when reproducing the Contribution or extracts from it, the Authors acknowledge first and reference publication in the Journal, the Authors retain the following non-exclusive rights:

a) To reproduce the Contribution in whole or in part in any printed volume (book or thesis) of which they are the author(s).

b) They and any academic institution where they work at the time may reproduce the Contribution for the purpose of course teaching.

c) To reuse figures or tables created by them and contained in the Contribution in other works created by them.

d) To post a copy of the Contribution as accepted for publication after peer review (in Word or Text format, not the NPG PDF) on the Author's own web site, or the Author's institutional repository, or the Author's funding body's archive, six months after publication of the printed or online edition of the

NPG encourages the self-archiving of the accepted version of your manuscript in your funding agency's or institution's repository, six months after publication. This policy complements the recently announced policies of the US National Institutes of Health, Wellcome Trust and other research funding bodies around the world. NPG recognises the efforts of funding bodies to increase access to the research they fund, and we strongly encourage authors to participate in such efforts.

Authors wishing to use the published version of their article for promotional use or on a web site must request in the normal way. For more info, please see the author guidelines as listed on the webpage link below: http://npg.nature.com/npg/servlet/Content?data=xml/05_news.xml&style=xml/05_news.xsl OR Visit http://npg.nature.com and click on the link "For

Authors" for the Author License. If you wish to distribute the NPG PDF in any way other than that described in the above link, you will need to purchase e-prints. E-prints are supplied as an encrypted PDF which can be posted on your website for e-print downloads. Each PDF can be viewed and printed *once* by the end-user. This secure document delivery system allows us to provide encrypted PDFs for posting on commercial websites, for a specific number of views/prints or e-prints. The PDF simply installs a one-time browser-based plug-in to your Acrobat Reader.

You can order your desired quantity and reorder at any time. Please visit our webpage at http://www.nature.com/reprints/permission-requests.html. Please let me know if you have further questions.

Kind regards,
Charlotte Kinnah
Permissions Assistant
nature **p**ublishing **g**roup
The Macmillan Building 4-6 Crinan Street
London N1 9XW
permissions@nature.com
+ 44 207 014 4129 f: + 44 207 843 4998

From: Pankuri Goraksha-Hicks [mailto:gora0042@umn.edu]
Sent: 25 November 2008 14:24
To: Permissions@nature.com
Subject: Re: need copyright permission letter

Hello Charlotte,
I am mainly looking to use some of the figures and the tables for the thesis.
Thanks!

Pankuri

On Nov 25, 2008, at 4:17 AM, <Permissions@nature.com> wrote:
Dear Dr. Goraksha-Hicks,
Thank you for your permissions request. Are you looking to use a figure or the full artilce to use inside your thesis?
Many thanks and kind regards,

Charlotte Kinnah

Permissions Assistant, nature **p**ublishing **g**roup

From: Pankuri Goraksha-Hicks [mailto:gora0042@umn.edu]
Sent: 24 November 2008 21:07
To: Permissions@nature.com
Subject: need copyright permission letter

Hello

I am preparing my doctoral thesis at the University of Minnesota and would like to include material from the following publication of which I am the first co-author:

Eunjung Kim, Pankuri Goraksha-Hicks, Li Li, Thomas P Neufeld, Kun-Liang Guan (2008), Regulation of TORC1 by Rag GTPases in nutrient response. NCB vol 10(8) pp. 935-45.

I am required to obtain a letter of permission from the publisher, authorizing the use of material for publication in my thesis. This is my second attempt at getting in touch with you regarding the same. The University prefers a letter of permission as against an online permission. Kindly let me know how I can avail such a letter as soon as possible.

Regards,

Pankuri Goraksha-Hicks

gora0042@umn.edu